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Comparative Proteomic Analysis Of The Impact Of Management Programs On Porcine Airways

Chakia Joi McClendon
North Carolina Agricultural and Technical State University

Comparative Proteomic Analysis of the Impact of Management Programs on Porcine Airways

Chakia Joi McClendon

North Carolina A&T State University

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Chakia Joi McClendon

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Approved by:

_____	_____
Dr. Jenora T. Waterman Major Professor	Dr. Sang-Hyon Oh Committee Member
_____	_____
Dr. Niki Whitley Committee Member	Dr. Gregory Goins Committee Member
_____	_____
Dr. Ralph C. Noble Department Chairperson	Dr. Sanjiv Sarin Associate Vice Chancellor for Research and Dean, School of Graduate Studies

Dedication

First and foremost, I thank God for using me as a model for future students who believe and want to journey on this career path. I strongly dedicate this research and thesis to my major advisor, Dr. Jenora Waterman, who has and continues to give me the support and encouragement to carry on and to reach for my goals. My upmost appreciation goes to my family and friends for keeping me focused and allowing me to follow my ambition.

Biographical Sketch

Chakia Joi McClendon was born May 03, 1984 in Stamford, Connecticut. She received a Bachelor of Science degree in Animal Science from North Carolina Agricultural and Technical State University in 2006. She is a candidate for the Master's of Science degree in Animal Health Science.

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List of Abbreviations

2D	Two-Dimensional
AmphoB	Amphotericin B
ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
CAFOs	Confined Animal Feeding Operation
COX-2	Cyclooxygenase-2
DE	Dust Extract
ECL	Enhanced Chemiluminescence
HBSS	Hank's Buffered Salt Solution
IEF	Isoelectric Focusing
iNOS	inducible Nitric Oxide Synthase
IPG	Immobilized pH Gradient
kDa	kilodaltons
LPS	Lipopolysaccharide
ml	milliliter
RPM	Revolutions Per Minute
Pen-Strep	Penicillin-Streptomycin
PBS	Phosphate Buffered Saline
PTE	Porcine Tracheal Epithelial
SCF	Swine Confinement Facilities
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SFM	Serum Free Medium
TBS-T	Tris Buffered Saline-Tween

Abstract

In North Carolina, swine are reared in confinement housing or pasture-based operations and air quality in confinement operations is known to contribute to health status. However, few if any reports investigate the impact of swine management operations on porcine airways; thus the goal of this project. The hypothesis was that airways of pigs reared indoors have morphological and proteome differences compared to pigs reared outdoors. Three experimental trials were conducted to observe airway morphology and proteomes. Trial I included three breed types raised indoors, Tamworth X Berkshire, Berkshire X Berkshire and Hertford X Berkshire (n = 4-5 each). Trials II and III consisted of animals reared in both environments; Trial II had 28 pigs (n = 14 each, indoor and outdoor) and Trial III had 48 pigs (n = 24 each, indoor and outdoor). For Trial III, body weights were recorded weekly for seven weeks to adjust tracheal measure for body size. Two airway morphology features, total tracheal and lumen diameters, were recorded and compared in all trials. One-way and two-way analysis of variance (ANOVA) was performed and LSmeans with the PDiff Option was used to separate means as applicable. Outdoor animals had a larger variation of body weights than indoor animals; however, there was no correlation between tracheal measurements and body weight in this study. There was a difference in airway diameter and lumen among animals reared indoors versus outdoor (p-value < 0.05); however, pens within housing type may have an effect. Comparative proteomics results suggest there are subtle differences among airway epithelia of animals reared indoors versus outdoors. Porcine airway epithelial cells exposed to swine confinement facility dust extract *in vitro* showed differential proteome modulation, including key inflammatory mediators cyclooxygenase-2 and inducible nitric oxide synthase. Taken together, these results demonstrate that there may be subtle differences between the impacts of the two hog management styles on porcine airway epithelial proteomes and morphology.

CHAPTER 1

Introduction

North Carolina is the second largest pork producing state in the America. Between the years of 1988 and 1997, the state of North Carolina established advantages within the swine industry by increasing pig and pork production at a rapid rate and maintaining its production through new management styles. Swine production currently generates about 25 percent of North Carolina's gross farm receipts, indicating it to be an essential component of North Carolina's economy (USDA Census of Agricultural, 2007).

Pigs can be raised in a controlled or free environment. The two types of swine management operations common in North Carolina are identified as commercialized indoor operations, and pasture–based outdoor operations. The majority of traditional swine producers have converted to modern swine confinement buildings so farmers are able to facilitate climate control and automate small tasks such as feeding and watering (Cole, Todd et al., 2000). Having the ability to control the pigs' environment allows farmers to standardized their management applications and enhance animal performance (Plain and Lawrence, 2003). Outdoor, pasture-based operations are usually described as free range settings, where pigs are able to roam and explore the outdoor environment. Animals maintained outdoors need to have a robust body composition in order to endure changing climates, exhibit appropriate behavior and deal with social competition for resources of feed or shelter (Edwards, 2005). At the North Carolina A&T State University Farm-Swine Research Unit, animals are raised within both types of environments. In the United States, 5% of gestating sows are kept outdoors. An additional 15% of US gestating sows are housed in buildings with outdoor access (Honeyman, 2005). This indicates that 85 – 95% of sows are housed indoors; however, there have been reported cases of

reduced quality of life as well as health problems related to airborne emissions from confinement housing (Wing and Wolf, 2000).

Indoor operations are known to significantly increase the susceptibility of airway inflammation and tissue damage during both short and long term exposure when inside or nearby these facilities (Iversen, Kirychuk et al., 2000). The indoor atmosphere in swine confinement buildings contain gases, dust and endotoxins in concentrations significantly in excess of those in outdoor environments. Research has shown repeated exposure of organic dust can orchestrate inflammatory responses, which have been implicated through an increased morbidity of exposed subjects (Poole, Alexis et al., 2008). It has been known for decades that agricultural workers are exposed to materials such as organic dusts, allergens, infectious agents and toxic gases which can induce severe lung disease. The populations at risk include farm workers, the outsiders who come in to perform their contracted duties (e.g., veterinarians), animals that are housed in these particular areas, as well as local community residents surrounding the agricultural land. Some distinctive syndromes and diseases that are typically associated with organic dust exposure include occupational asthma, chronic bronchitis and chronic obstructive pulmonary disease.

Observing airway epithelium is an important portion of this research as epithelial tissue lines the trachea and can act as a physical barrier and as a regulator of physiological and pathological events in the respiratory system. Damage to this airway barrier by oxidants released from inflammatory cells is a key feature in respiratory diseases such as bronchial asthma (Truong-Tran, Grosser et al., 2003). As stated above, in certain human populations, swine confinement facility (SCF) exposures have been associated with airway respiratory diseases. Therefore the impetus for this work is to determine if differences exist within the airways of pigs reared indoors compared to outdoors.

A proteomics approach was used to identify potential differences in tracheal epithelial proteins from animals reared indoors and outdoors. Within the two environments, we were able to analyzed proteins profiles of pig populations and report how those subtle differences exist. This work can give insight for understanding the cellular mechanisms that occur in the airways of confinement animals, and thus, contribute to the body of knowledge concerning the impact of air quality in swine confinement operations on respiratory health.

CHAPTER 2

Literature Review

Pork production is a multibillion dollar industry in the United States, and it is a significant contributor to the agricultural economy in North Carolina, the second largest pork producing state in the nation. Therefore, the health and welfare of pigs is an ongoing concern for pork producers. It is well accepted that the respiratory anatomy and physiology of pigs is closely related to that of humans. Most of the time the causative factors that result in symptoms of poor health in humans, may manifest similar problems in pigs. Food animals in particular are our main focus because these animal populations are the center of mass production efforts and are utilized for consumable products.

The increase in animal production during the past 50 years has led to massive changes in the husbandry of pigs. This has resulted in the transformation of outdoor facilities to confinement housing for mass production of hogs (Hötzel, Pinheiro Machado F, et al., 2004). In recent years, there has been a public fear for the health of the employees as well as the neighbors, who live in nearby communities of confined animal feeding operations (CAFOs). In particular, the concern is focused on the possibility that workers could develop airway diseases such as asthma or asthma-like syndrome and chronic bronchitis. Occupational studies of workers at these facilities have reported a variety of health complaints and effects including respiratory inflammation and dysfunction (Schiffman, Studwell et al., 2005). This type of environment can be hazardous to the animals' health as well. CAFOs are known to produce indoor aerial contaminants including gases, particulates and airborne microorganisms (Kim, Ko et al., 2005). Gaseous and particulate contaminants are typically derived from the pig, feed and dried manure and are distributed into the air as dust particles that may become inhaled by the animal or human (Kim, Ko et al., 2007).

Some of the biological contaminants in pig confinement housing include airborne viruses, bacteria, fungi and endotoxins such as lipopolysaccharides (LPS) (Crook and Sherwood et al., 1997). Animals in outdoor production facilities have been shown to contain a bacterial diversity as well. However, little to no studies have been performed to identify the impact of both environments/management styles on the airway epithelia of pigs. A brief summary of current literature related to confinement and free-range swine management operations will follow.

Swine Management Operations

There are two types of hog management operations: commercial (indoor) and pasture-based (outdoor). A great number of pigs are raised indoors in confinement buildings in the United States, Europe and elsewhere. This method of raising pigs has economic advantages, primarily by reducing labor costs and by decreasing time to market readiness. Thus, confinement rearing has become common practice during the past 20 years (Nation Ag. Safety database, 2001). On large farms, caring for pigs housed in these types of facilities, which are also known as swine confinement facilities (SCF), is a full-time occupation for the owners and/or employees of the farm. It has been estimated that in the United States, 250,000 people work in swine confinement facilities (Nation Ag. Safety database, 2001). These persons include workers responsible for the daily care of the animals, veterinarians and individuals that perform specialized operations such as cleaning the farm buildings. Research has shown for over 20 years that exposure to the commercialized operations can cause acute and chronic respiratory symptoms in many of the workers who care for the pigs (Preller, Heederil et al., 1995 and Mathisen, Von Essen et al., 2004), largely due to exposure to high concentrations of respirable dusts and gases (Bailey, Meza et al., 2007). The symptoms either represent an asthma-like syndrome, chronic bronchitis, or exacerbation of pre-existing asthma.

Figure 2.1 depicts the two management programs that will be discussed within this section. These two images represent an example of modern commercial/indoor (figure 2.1A) and traditional pasture-based/outdoor (figure 2.1B) operations. In the indoor setting, there is a visible accumulation of particulate material on the ground and on top of railings; usually the build-up of dusts, manure and organic material. The outdoor image shows a free range setting, where animals can be exposed to a variety of environmental factors. These images were taken at the North Carolina A & T State University Farm-Swine Research Unit. For the current study, animals were housed either indoors or outdoors for at least seven weeks.

Figure 2.1. Swine management operations.

Confinement Operations

It is estimated that 90% of pigs in the United States now live their entire life, of approximately 24 weeks for market pigs, and longer for breeding stock indoors (Plain and Lawrence, 2003). In general, indoor facilities have an arranged set of production steps and, as a result, one building does not work alone and is considered part of a system. The sequence of production phase buildings (or facilities) includes breeding (gestating), farrowing, nursery, growing and finishing units. In this type of management system animals are able to be

maintained inside until they reach a market weight of 108.9-122.5 kilograms, which is approximately 20-24 weeks in time (Holden and Ensminger, 2006).

An advantage to indoor facilities is the ability to engineer adequate buildings and to have environmental control. The United States is the second largest pork producer globally following China and the second largest exporter after Denmark (Agricultural Marketing Resource Center, 2012). With these types of management styles farmers are capable of producing over 50,000 pigs annually (Pelletier, Lammers et al., 2010). Therefore, properly designed houses or farming units are extremely imperative when raising animals for the market. Commercialized agricultural operations must take into account proper shade, insulation, ventilation, heating and air conditioning in order to maintain maximal hog production performance.

The increased demand of confined, environmentally controlled, remote unit approach for managing and raising swine has been continuously adopted. This adopted style has provided improved working conditions for the caretaker of the animals and, at the same time, is assumed to result in greater growth and productivity of these animals. Ventilation systems have been an innovative method for eliminating air pollutant concentration and maintaining proper environmental conditions within the confinement facility. Also, controlling the temperature and the humidity indoors for swine is essential, because these factors may affect their growth performance. For producing swine, the optimum air temperature is 10-15.6°C and the humidity should be at 50 to 80 percent (Miller, 1976). At higher temperatures, hogs do not eat as much feed, causing less weight gain over time. In contrast, at lower temperatures, some of the feed must be used to produce body heat and consequently weight gain is also reduced. Therefore, having control of the indoor setting is vital to produce maximal growth of animals.

However, ventilation of confinement facilities has many challenges. Currently, ventilation systems are being heavily investigated (Topisirovic and Radivojevic, 2005). During the last 20 years, research of indoor agricultural facilities has increased tremendously. Investigations and analyses are ongoing with regard to confinement housing because of the possible associated harmful effects. Costal et al. (2009) selected swine houses with ventilated controlled systems to evaluate the contribution of the various compartments of swine husbandry to dust and polluted emissions into the indoor atmosphere during one year of observation. Results showed the highest concentration of particulate matter and emission was found within the fattening piggery compartment as well as the particulate matter reaching its maximum values during feeding times (Costal and Guarino, 2009). Another study was conducted to evaluate the ventilation rate and its impact on aerial contaminant within confinement pig buildings (Kim, Ko et al., 2007). Multiple air samples were taken over a 90 day period and analyzed at different rates of ventilation. It was found that gaseous contaminants, like ammonia and hydrogen sulfide, decreased as the ventilation rates increased. However, concentrations of total dust and total airborne microorganisms were not affected by ventilation rates. The implications of this study suggest that by means of the size and weight of dust particulates and airborne microorganisms may be possible reasons for the failure of ventilation systems to eliminate these types of aerial contaminants from indoor systems (Kim, Ko et al., 2007).

Although ventilation is present in these facilities, human and animal exposure to agricultural dust, soil particulate matter and animal manure still exists at high levels. Indoor environments are more susceptible to accumulation of these pollutants, possibly causing respiratory illness within the airway of workers such as asthma, chronic obstructive pulmonary

disease and chronic bronchitis. It would thus stand to reason that outdoor operations would generate less particulate build-up since they are more open systems.

Pasture-based Operations

Outdoor swine management styles are perceived as more humane, environmentally friendly, traditional and a sustainable process of rearing pigs (Edwards, 2005). However, outdoor operations involve the risky factor of poor air quality too. In an outdoor environment, animals are met with far more involuntary challenges, such as unfavorable weather conditions. In the current study for example, pigs from Trial III were raised during the spring, between the months of April and May. And in these months, increased rainfall is common for the southeast. Other factors animals must endure while outdoors is the stringent exposures of pollen, dirt, manure and dust.

There are several differences between the two management styles. One difference is that pigs within the outdoor systems have the potential to scavenge for a range of different feedstuffs. Tajima et al. (2010) conducted a study to evaluate the bacterial community in the feces of pigs in an outdoor production system. The control animals were located within an indoor production system. Results showed bacterial diversity being higher with pigs reared outdoors when compared to the control group. This data suggest that pigs reared outdoors have the opportunity to be exposed to various environmental factors, such as soil, grass and other plant products (Tajima, Kobashi et al. 2010). So, unexplainable factors like individual differences between animals, seasons and diet conditions that may affect animal health within an outdoor system should be considered or taken into account.

Pasture-based swine production overall has an increased environmental diversity and behavioral freedom. However, outdoor pigs may encounter greater welfare problems such as

increased pathogen contact and temperature stress (Gourdine, de Greef et al., 2010). It is suggested that genetic improvement in health and welfare of animals maintained outdoors is needed, especially in good mothering ability, strong legs, and sow longevity (Hirt, Bestmann et al., 2001; Held, Manson et al., 2006; Leenhouwers, Napel et al., 2009). With an open environment, animals are able to roam and explore at their own leisure, having the potential for both positive and negative effects on the airway. Plentiful space and relatively fresh air may reduce or increase infection or signs of respiratory illnesses in these animals. By evaluating the trachea and the airway epithelium of the pigs, our interest is to detect and distinguish those structural and proteomic differences potentially influenced by the two management styles.

The Anatomy of the Trachea

The respiratory system consists of upper and lower respiratory tracts. The upper respiratory tract includes the nasal cavities, pharynx and larynx. The trachea, the bronchial tree and the lungs comprise the lower respiratory tract (Mader, 2010). The trachea in particular is a very dynamic organ, having the ability to conform during respiratory cycles when breathing (Russo, Robinson et al., 2008). In addition to physical change, the internal portion of the trachea can exhibit other physiological changes due to environmental responses.

The trachea is commonly known as the windpipe, a tube-like organ that extends between the larynx and the primary bronchi. It is supported by smooth muscle and connective tissue, in which cartilage rings are suspended. These discontinuous cartilaginous rings, or 'c-rings' as they are commonly called, are present to prevent the trachea from collapsing. The trachea is located anterior to the esophagus, where it is separated by a flexible muscular wall called the trachealis muscle. This placement facilitates the consumption of food when swallowed, as the esophagus is able to expand without interruption (Fox, 1987).

The main function of the trachea is to transport conditioned air into the lungs for gas exchange as well as to capture foreign particles and move them upwards, away from the airway. Coughing occurs as a result of irritation in the trachea and bronchi. During this time the tracheal wall can contract, narrowing its diameter. When particles are too small to trigger a cough they can become trapped in mucus produced by mucus-secreting goblet cells within the epithelial tissue lining these structures (Belk, Collen and Borden, 2009).

In response to environmental irritants, air pollutants, allergies and other airway inflammatory triggers, airway epithelia cells can elicit an over-production of mucus. Excessive mucus production is often observed during airway inflammation and is one of the leading causes of airway obstruction in asthma and chronic bronchitis (Wang, Wen et al. 2007). In the current study, several parameters of airway anatomy were evaluated, including total tracheal and lumen diameters. Due to the dynamics of the trachea, this organ is assumed to have subtle yet structurally distinct differences within pigs reared indoors when compared to pigs reared outdoors. Airway responsiveness may also be different according to the animals' environment and/or genetic background.

Tracheal Epithelium

The entire tracheal tree is covered with continuous epithelial cells that are essential for maintaining the normal function of the respiratory system (Berube, Prytherch et al. 2010). Airway epithelia have multiple specialized responsibilities that include: forming barriers to a wide range of physical or chemical abuse, facilitating mucociliary clearance, producing protective secretions, repairing and regeneration abilities, and mediating the response of other airway components like inflammatory cells (Proud, 2008).

In the present study, the lower respiratory tract was the focus, particularly the trachea, which is lined primarily with pseudostratified ciliated columnar epithelial cells, mucus (goblet) cells and basal cells. Pseudostratified ciliated columnar epithelial cells are the most abundant of the tracheal cell types. Cilia are commonly defined as short, hair-like structures projecting from the apical surface of the epithelium. Their function is to provide a coordinated sweeping motion of the mucus blanket toward the pharynx. This process is known as mucociliary clearance, which serves as an important protective mechanism for removing small inhaled particles from the lungs. Mucus cells are distributed among the ciliated cells and can increase in number during chronic irritation of the airways. Basal cells are reserve populations that supply individual cell replacement in the epithelium (Ross and Pawlina 2006).

In addition to the protective barrier formed by the epithelium lining the respiratory tract, cells of the epithelium also have the ability to adapt to stress as another defense mechanism. Metaplasia can also occur, in which the cells can be replaced by another cell type (Kumar, Abes et al. 2010). Individuals with chronic exposure to noxious inhalants experience epithelial change occurrence. When the cilia on the ciliated cells lose their function, the removal of mucus is impaired. To compensate, coughing typically occurs as a secondary mechanisms to remove foreign particles from the airway. Over time, the number of ciliated cells becomes reduced due to the chronic coughing. The loss of ciliated cells further damages the normal epithelium and results in replacement with another epithelium cell type. This change or adaptation is known as metaplasia (Ross and Pawlina 2006).

In maintaining homeostasis, epithelial cells not only exhibit mucociliary clearance, secretion of ions and regulation of the airway surface liquid water content, but can also produce inflammatory/anti-inflammatory proteins, bacterial/antibacterial constituents and

oxidative/antioxidant molecules in the mucus (Chang, Shih et al. 2008). Therefore, in the present study, a proteomic approach was taken to identify molecules within the airway epithelial tissue of animals reared indoors versus those reared outdoors. This will provide insight into distinguishing differences between the two management styles and their impact on the airway epithelium, which has yet to be established, as well as biological mechanisms that occur within the mammals that are exposed to the two swine production operations.

In response to inhaled endotoxins or organic dust in swine confinement buildings, different types of protective mechanisms can occur. Human exposure to endotoxin has been implicated as a principal pathogenic agent in several occupational like asthma and chronic bronchitis. Chronic exposure to a noxious environment can compromise the defense system, causing the airway epithelium to be repeatedly injured. After airway cell injury, the basement membrane can be partially or completely stripped of basal cells, with these common changes in structure and function playing a critical role in the development of respiratory complications (Coraux, Hajj et al. 2005, Knight and Holgate et al. 2003).

Inflammatory Airway Diseases

Agricultural workers are potentially exposed to a variety of gases as well as various organic and inorganic dusts. Pig farmers and swine confinement workers have an increased risk of chronic bronchitis and asthma-like syndrome. These inflammatory airway diseases may develop following exposure to high concentrations of an irritant gas or dust. Chronic cough and chronic bronchitis in swine producers has been related to endotoxin levels measured in swine confinement buildings. Current research has found evidence of inflammation, characterized by increased numbers of neutrophils, macrophages and lymphocytes, in both non exposed subjects and swine confinement facility workers (Von Essen and Romberger 2003).

Animal health is an issue as well. Animals with chronic airway exposures can possess poor respiratory health status, which can make them more susceptible to devastating respiratory diseases such as Porcine Respiratory Reproductive Syndrome (PRRS) or swine influenza. As of 2005, the prevalence of PRRS within the United States was estimated at 60 to 80 percent (Baysinger et al. 2005). The main clinical effect of PRRS is reproductive failure and respiratory distress in pigs of all ages. Stress factors, such as confinement housing, can reduce the efficient immunological responses of pigs that usually prevent pathogenic diseases from occurring.

Swine housing is one main factor to consider when evaluating the respiratory health of animals. Certain causes of stress within the production environment can potentially have suppressive effects on the immune system, thereby increasing susceptibility of animals to infectious diseases. Sutherland et al. (2007) investigated heat stress and social rank on immune responsiveness and performance of confinement facility pigs challenged with the virus that causes PRRS. A total white blood cell count was collected to evaluate immunological responses. Results showed that both heat stress and social rank did have an impact on the pigs' immunity and performance (Sutherland, Niekamp et al. 2007). Thus, understanding the underlying impact of dust as a stressor on the airway of pigs will provide insight on the biological mechanisms that occur and may cause weakened immune systems of these animals.

To our knowledge, little to no studies have investigated the direct impact of management environment on porcine airway epithelial tissue. Although animals are generally less affected, it is suspected that differences exist in the airways of pigs reared indoors versus outdoors. The exorbitant knowledge base is generated by evaluated potential respiratory distinctions. Due to the similarities in respiratory anatomy and physiology, human respiratory health research studies have direct connection to the animals that live in these types of environments. Therefore, the

hypothesis is that the airways of pigs reared indoors have morphological and proteomic differences compared to those reared indoors.

Research Objectives:

1. Compare airway morphologies of tracheal regions from pigs reared indoors and outdoors.

2. Evaluate the effect of genetic background and body weight on airway morphologies.

3. Determine *in vivo* basal protein levels of tracheal epithelia from pigs reared indoors and outdoors.

4. Characterize airway epithelium proteomes of pigs reared indoor and outdoor.

5. Determine airway epithelial cell function in pigs reared indoor and outdoor using an *in vitro* model.

CHAPTER 3

Materials and Methods

Animals, Housing and Experimental Design

The North Carolina Agriculture & Technical State University Institutional Animal Care and Use Committee approved all experimental procedures involving animals in this study. The Swine Research Unit includes a 250-sow indoor commercial hog-rearing environment, as well as two hoop barns, and a pastured hog operation for small-scale or limited-resource farmers making the transition to hog farming from tobacco or other crops.

Trial I: Impact of indoor rearing study. The objective of Trial I was to evaluate the impact of indoor rearing on airway morphology and airway epithelial proteomes using three different breed types. Fifteen crossbred pigs (Tamworth X Berkshire (TB), Berkshire X Berkshire (BB) and Hertford X Berkshire (HB)) were selected from North Carolina A&T State University Farm-Swine Research Unit. Trial I was conducted from September 2009 through March 2010. Pigs were maintained in environmentally controlled confinement housing pens with ad libitum feed and access to nipple drinkers. At approximately five to six months of age, pigs were transported to a USDA approved abattoir for harvest. Only fourteen tracheas were collected; one was obliterated during the dissection process and could not be used on study. The crossbred tracheal samples collected were grouped as follows; TB (n = 5), BB (n = 5) and HB (n = 4; one trachea was obliterated during harvest and was not used).

Trial II: Impact of indoor and outdoor rearing study. The objective of Trial II was to evaluate the impact of confinement versus pasture-based rearing on the airways. Twenty-eight pigs, born and weaned from a confinement unit were reared in a pasture-based setting during the months of June through November 2010. At approximately five to six months of age, pigs were

transported to a USDA approved abattoir for harvest. All tracheal samples collected were evaluated as described below and stored for further processing. However, for Trial II, only fourteen of the outdoor tracheal samples were selected randomly and used. The fourteen tracheas that were collected from Trial I were used within trial II to compare the differences between the two management styles and its impact on airway morphology and airway epithelial proteome.

Trial III: Impact of rearing style and potential confounding factors study. The objective of Trial III was to investigate the impact of management style on porcine airway dynamics while controlling for variables such as body weight, breed and sex. Forty-eight crossbred piglets were selected (primarily Yorkshire and Landrace crossbred) from North Carolina A&T State University Farm-Swine Research Unit. The trial was conducted from April through May 2011. After the weaning period (of three weeks), twenty-four piglets remained within an indoor production setting and twenty-four were placed within an outdoor production setting. All animals on study were given numbered ear tags for identification. Body weights were observed and documented weekly over a seven week period. Final body weights were recorded on the day of sacrifice at approximately seven weeks of age.

Within the indoor setting, eight pigs were assigned to three different nursery rooms. Pigs were maintained in 1.2192 X 3.048 meters pens with four pigs per pen within the nursery rooms, in which were environmentally controlled and maintained at 22.2 to 25.6°C. Pigs were fed 8.2 kilograms of a normal NRCS-based growing pig diet daily and had access to nipple drinkers. The nursery pens had metal slats that allowed for manure disposal.

Within the outdoor setting, pigs were divided into three pasture areas (15.24 X 28.956 meters) with eight pigs each. Pigs were fed 10.9 kilograms of a normal growing feeding diet daily and had access to nipple drinkers. The trial lasted seven weeks. Figure 3.1 shows the

experimental design of the two management environments used in Trial III. Animals were kept in their designated areas for seven weeks. The outdoor animals (left) were provided with feed (F), English Hut for shelter (S) and water (W). Indoor pigs (right) were separated further into two groups of four in adjacent pens with their own feeder and water (F).

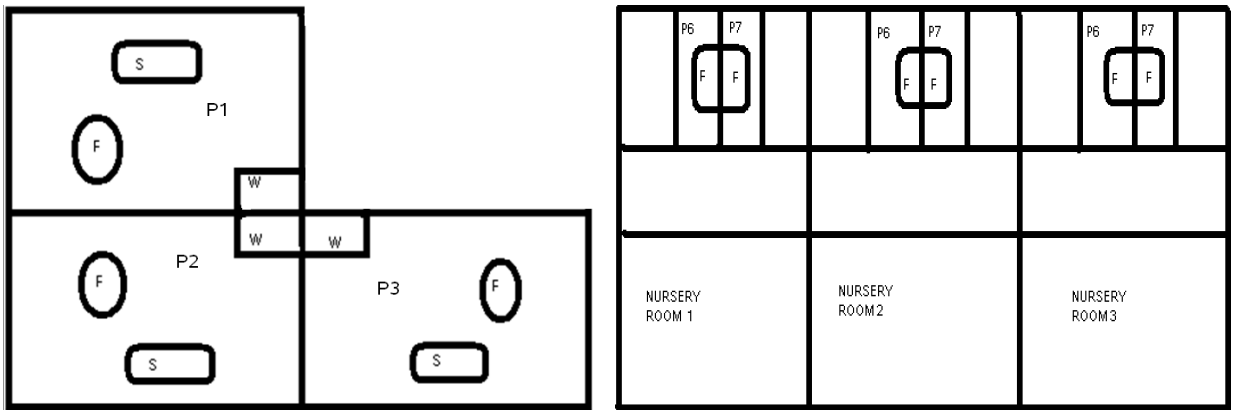


Figure 3.1. Outdoor and indoor setting for Trial III.

Porcine Airway Acquisition and Analysis

Pigs were provided by Dr. Sang-Hyon Oh, Department of Animal Sciences, North Carolina A&T State University. At a body weight of at least 19.1 kilograms, animals were harvested. At the end of each trial, animals were transported for harvesting at a USDA-approval abattoir.

At sacrifice, the tracheal portion of the respiratory tract was dissected using a clean blade. One at a time, the whole respiratory tract was collected, then tracheal samples were dissected by severing the connective tissue beneath the larynx (anterior to the first cartilaginous ring of the trachea) and directly above the point of bronchial branching into the lungs. Excess connective tissue, lymph nodes, heart and lung tissue were removed from specimens using a scalpel. After dissection of the trachea, each section was then placed into ziptop plastic bags and kept on wet

ice during transportation. Samples were returned to the laboratory where airway measurements were collected.

Airway measurements. On the day of harvest, five parameters were measured: tracheal length, tracheal (outside) diameter, tracheal (inside diameter) lumen, whole tracheal weight and one (Trial I and II) or two (Trial III) inch dissected section tracheal weight. Figure 3.2 displays a model of the measurements recorded during this data collection. Tracheal length was measured from the top of the arrow right below the larynx to the beginning of the principal bronchus (Fig. 3.2A). Tracheal Diameter was measure in two directions on a longitudinal and latitudinal plane (Fig. 3.2B). Tracheal lumen was measured in two directions, similar to the tracheal diameter. However, this pertains to the airspace within the tracheal (Fig. 3.2C). Whole tracheal weight was weighed before a one to two –inch segment was removed and weighed (Fig. 3.2D). One/Two-inch section was removed from the distal end of the windpipe and stored for further processing (Fig. 3.2E). The dissected segments were needed for epithelial tissue protein isolation. The extra half of the trachea was utilized for isolation of airway epithelial cells.

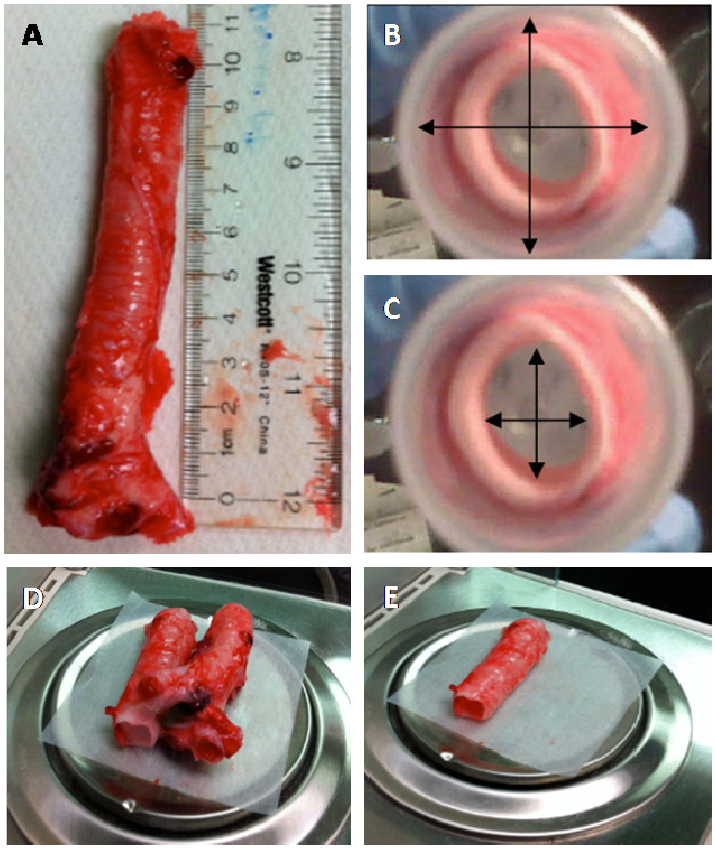


Figure 3.2. Images of tracheal samples for airway measurements.

Airway Epithelium Protein Isolation and Quantitation

Tracheas previously stored at -80°C were allowed to thaw on wet ice for 15 to 20 minutes. During the thawing period, a protein extraction buffer (Appendix A) was prepared. Each epithelial tissue layer was removed, weighed and diluted with an extraction buffer (0.5 grams of epithelia tissue/1 ml of extraction buffer). The protein extraction buffer included; 10mls of 10X RIPA lysis buffer solution and 90 mL of dH_20 (Millipore, Temecula, CA), one tablet of Complete Protease Inhibitor Cocktail (Sigma-Aldrich, ST. Louis, MO) in a 5 ml solution of 1X

RIPA (1X) lysis buffer, a protease inhibitor (Sigma-Aldrich, ST. Louis, MO) and a phosphatase inhibitor (Sigm-Aldrich ST. Louis, MO).

A clean scalpel blade was used, as each trachea was processed separately to cut the one/two-inch tracheal segment longitudinal into a open spread. The same scalpel blade was then used to scrap and recover the inner epithelial lining of the airway. Approximately 0.5 grams to 1.5 grams of epithelial tissue was weighed then placed into the extraction buffer (0.5g/1ml solution) to be disintegrated into smaller fragments with a Brinkman Homegenizer (PT10/35). The sample was ground for 10 seconds on setting #9 (equivalent medium to high speed) and then incubated on wet ice for 10 seconds to keep cold. These steps were repeated three times to ensure the isolation of proteins. After homogenization, the sample was then centrifuged for 30 minutes in 4°C at the speed of 3,220 xg (IEC Centra – 7R refrigerated centrifuge). After centrifugation, the supernatant was recovered and placed into 1.5 ml eppendorf tube and centrifuged for another 30 minutes in 4 °C at the speed of 20,124 xg (Beckman Microfuge R Centrifuge, Palo Alto, CA). this step was repeated, and the final supernatant was recovered and stored at -80°C until further investigation (Appendix A).

Quantitation of isolated proteins. A Bradford Protein Assay was used to measure the concentration of proteins in the epithelial cell supernatant from the protein isolation. Standard solutions used in all of the calibration curves were obtained after the correct dilution of Bovine Serum Albumin (BSA) was prepared (0, 50, 100, 200, 400 and 800). Bradford Reagent Assay Dye (Bio-Rad, Hercules, CA) was prepared by a dilution ratio of 1:4 with dH_2O. Unknown protein samples were prepared in triplicate using dilution ratios of 1:100, 1:200 and 1:300 using dH_2O. Diluted concentration BSA standards (10 µl) were then pipetted into 12 wells. The diluted epithelia protein samples (10µl) were added to 96-well assay plate (Corning, Corning, NY) in

triplicate. Bradford Assay Dye (190 µl) was added into each occupied well. The samples were read using a VersaMax Microplate Reader (Molecular Devices, Sunnyvale, CA) at 595 wavelengths. Protein concentration values were determined by SoftMax Pro 5.3 (Molecular Devices) (Appendix A).

Protein Analysis Via Gel Electrophoresis-based Proteomics

Two-dimensional (2D) gel electrophoresis is a valuable tool for proteomics. It is a technique used to separate proteins into two dimensions as opposed to one dimension. Researchers utilize this tool for high-resolution profiling for low abundance proteins. The first step includes proteins being separated by isoelectric focusing (IEF). All proteins applied to the IPG strip (4-7 pH gradient) in the first dimension will move along the gel strip and will accumulate at their isoelectric point; that is, the point at which the overall net charge on the protein is a neutral charge of zero. The proteins are then applied to the second dimension of separation, the sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) which runs in a perpendicular direction. This procedure generates an array of spots, each representing a protein. Individual protein spots may be excised and identified via mass spectrometry.

Two-dimensional gel electrophoresis. In the present study, an equivalent amount of protein extract was collected from four separate tracheal samples from the same porcine management operation as well as pen (Trial III, n=1 outdoors and n=1 indoors) and pooled by housing system. The pooled samples were mixed with the rehydration buffer having the maximum volume of 185 µl of solution mixture. The pooled solution was applied to a ReadyStrip^{TM} IPG strip, 11cm with a pH of 4-7 (Bio-Rad, Hercules, CA) and the strip was allowed to rehydrate (*i.e.,* absorb the protein-containing buffer) at 4°C overnight. The next day, proteins within the IPG strip were "focused" or separated in the first dimension (*i.e.,* based on

their isoelectric point) with a Protean IEF Cell model 526BR 08231 (BioRad, Hercules, CA) for approximately 6 hours, then stored at -80^{o}C overnight. The following day, equilibrium buffer I was placed on top of the gel strip for 10 minutes at room temperature with gentle agitation. Equilibrium buffer I was discarded and equilibrium buffer II was placed onto the strip for 10 minutes with gentle agitation. After draining of equilibrium buffer II, the 11 cm strip was affixed to a broad lane Criterion TGX any kD Precast Gel (Bio-Rad) with heated agarose gel. To separate proteins in the second dimension (*i.e.,* based on molecular weight) gel electrophoresis SDS-PAGE was conducted using a Criterion cell and Universal Power Pac HC (Bio-Rad) at 200 voltage for 65 minutes. Proteins were visualized via SimplyBlue^{TM} Safestain (Invitrogen, Carlsbad, CA) according to manufacturer's instructions (Appendix A).

In-gel tryptic digestion and liquid chromatography/mass spectrometry analysis. After electrophoresis**,** protein spots of interest were extracted from each gel and subjected to in-gel tryptic digestion. Tryptic digestion was performed via the 'Tryptic in-gel digestion of proteins' protocol (Proteomics and Mass Spec Core Facility, Huck Institutes, University Park; http://www.huck.psu.edu/facilities/proteomics-mass-spectrometry-up/sample-preparation). The trypsin (Promega; Sequencing Grade Modified Trypsin, Frozen) to protein sample ratio used was 1:20 and samples were incubated overnight for 18 hours at 37^{o}C. After incubation, samples were extracted from gel spots and speed-vacuumed to dryness. Peptides were purified and concentrated from organic solvents using PepClean C-18 Spin Columns (Pierce) according to manufacturer's protocol except centrifugation steps were performed using a USA Scientific Mini Centrifuge. Recovered peptides/samples were speed- vacuumed to dryness. Liquid Chromatography/Mass Spectrometry analysis of tryptic peptides and protein identification was

conducted at the Proteomics and Mass Spectrometry Core Facility, Huck Institutes at University Park, Pennsylvania State University.

Tracheal Epithelial Cell Isolation, Expansion and Stimulation

To evaluate the impact of swine management programs on airway epithelial cell function, cells were isolated, expanded to increase the number of cells and then stimulated with a dust extract.

Dissociation of airway epithelial cells. After the two-inch segment was removed from the top of the trachea, the remaining bottom half was used for tissue dissociation. Airways were cut into smaller (2.5 cm) rings and placed in sterile beakers with ice-cold wash media (Hank's Buffered Salt Solution, HBSS (Thermo), containing penicillin-streptomycin (Pen-Strep) (Milli-Pore), and amphotericin B (AmphoB). Airway rings were washed three times using wash media. Airway rings were cut opened with a scalpel and cut into 1 X 2 centimeter (cm) segments. Tracheal segments were transferred to 50 ml conical tubes containing 30 ml of dissociation media (DMEM with Pen-Strep & AmphoB) plus 4 ml of 10X Protease (0.1%)/ DNase (0.001%) solution. Approximate tissue to fluid ratio was 1:9, in a final volume of 40 ml. The airway segments were digested with 0.1% protease XIV and 0.001% DNase in dissociation media at 4°C for 24 hours with gentle agitation (on a platform rocker, 50-60 cycles/minute) (Appendix A).

Porcine airway cell isolation. From this point forward, sterile tissue culture techniques were used and tissues were processed using a Class II A/B3 Biological Safety Cabinet (Thermo Forma, 1286). The dissociation reaction was ended by adding heat inactivated Fetal Bovine Serum (FBS, Atlanta Biologicals, Atlanta, GA) to a final concentration of 10% (4 ml per tube). The tubes were inverted several times to neutralize the protease and immediately placed on ice.

Dissociation reaction tube contents were poured into a sterile 150-mm tissue culture dish one tube at a time. Cells were harvested by gently scraping internal surfaces of airway with a sterile surgical scalpel blade (BD Bard-Parker, BD Rib-Back Carbon Steel, No. 20) to slough off airway cells. The tissue surfaces and collection dishes were rinsed with ice-cold sterile tissue culture grade Phosphate Buffered Saline (PBS) and cell suspensions containing dissociated cells were pooled into solutions into 50 ml conical tubes and kept on ice. These tubes were centrifuged at 1250 rpm for 5 min at 4°C. Cells were washed, re-suspended in wash dissociation media (without protease/DNase), centrifuged again, and re-suspended in a small volume (3-5 ml) of Porcine Complete "Expansion" Media. The cell concentration was determined using a hemacytometer, and cell viability was assessed by trypan blue exclusion assay (Appendix A).

Porcine airway cell expansion. Cells were cultured using a method similar to Fuhrmann et al., (1999) and Muneta et al., (2002). The cell volume was adjusted to obtain a concentration of ~500 cells/cm^2 or 3.75-5.0 x 10^4 cells/ml. To ensure even distribution of cells, tubes (50 ml conical) were swirled gently prior to pipetting and following cell partitioning, flasks were checked with an EVOS microscope (Advanced Microscopy Group, Bothwell, WA). Culture flasks (75cm^2) were incubated at 37°C in humidified air containing 5% CO_2 overnight. The next day, media was removed and replaced with fresh media to remove non-adherent cells and tissue debris. The cells were allowed to proliferate for two days prior to the next media change. The cells were then fed Porcine Complete Expansion Media every other day until harvest. An expansion log was maintained to record cell growth and record confluency on a daily basis. When cultures reached 80-90% confluency (within 5-6 days), they were harvested and used immediately or stored in liquid nitrogen in 1.0 ml (2.0-2.5 x 10^6 live cells/ml) aliquots in freezing media until needed (Appendix A).

Growing porcine tracheal cells *in vitro*. For experimental investigations, six-well tissue culture plates (Corning, Lowell, MA) were labeled with name, date, plate number and donor identification number. Wells were coated with [50µg/ml] collagen type I rat tail (BD Biosciences) in 0.02 N acetic acid. Excess collagen was vacuum-aspirated off and washed with 1 mL of PBS for 10 minutes at room temperature. The PBS was vacuum aspirated and 2 ml of warm porcine complete media was added to each well. Frozen cells were retrieved from liquid nitrogen cryopreservation and warmed in a 37°C water bath for 1-2 min. The cells were added to pre-warmed media, and seeded at a density of 1.0 X 10^4 live cells/cm^2 on 6-well culture plates coated with rat tail collagen (type I). The plates were swirled gently to evenly distribute the cells and plates were incubated at 37°C overnight. The following day the media was changed and the cells were allowed to rest for two days. The cells were checked for confluency every day and the media changed every other day with Porcine Complete media until cultures were 85-90% confluent. Once confluency, was reached the cells were fed serum-free media (deficient in growth factors and FBS) for 24 hours to force cells into quiescence. After 24 hours, the cells were utilized for experimental investigations (Appendix A).

Preparation of swine unit dust extract (DE). Settled dust was obtained from raised surfaces at the North Carolina Agricultural and Technical State University Swine Confinement Facility. Samples were collected from the same locations each time; gestation, farrowing, nursery and breeding rooms. Settled dust from the fixtures of the pig pens was brushed into a Ziploc bag using a cosmetic brush and transported immediately to the laboratory for further processing. One gram of dust was added to 10 mL of HBSS and vortexed for one minute. The dust suspension was left to stand at room temperature for one hour. The mixture was centrifuged for 10 minutes at 5,000 rpm at room temperature. The supernatant was transferred to a new 50

ml conical tube and centrifuged again at 5,000 rpm for 10 minutes. The final supernatant was filtered by using 0.22 μm filter. The DE was used immediately (Appendix A).

Cell stimulation. Cells derived from animals reared indoor were stimulated with concentration gradients of DE, and lipopolysaccaride (LPS) was used as a positive control. The concentration gradient was as follow with an eight hour exposure time: control (media only), 1%, 2.5%, 5%, and 7.5% DE and LPS 100 ng/μl.

Preparation of whole cell extracts from PTE. Following DE exposure, treatment media was removed and cells/plates were placed on ice and cell surfaces were washed gently with 1 ml cold PBS (w/o Ca^{++} and Mg^{++}) containing phosphatase inhibitors (Active Motif, Carlsbad, CA). Ice cold extraction buffer (0.01%-protease inhibitor, 0.01%-phosphatase inhibitor and 98% of 1X Cell Signaling Lysis Buffer™) was added to each well, and plates were rocked on ice for approximately 30 minutes. A cell scraper was used to scrape cells to one side of the well. The cell suspensions were collected and transferred to microfuge tubes and returned to ice. The cell suspensions were sonicated three times for seven second bursts interspersed with 1 minute incubations on ice. The lysates were centrifuged at 15,000 rpm at 4°C for 15 minutes. The supernatant containing proteins was transferred to a new tube without disturbing the cell pellet. The protein concentration was subjected to a Bradford assay. Aliquots were stored at -80°C until needed (Appendix A).

Western blot analysis. For western blot analysis, sample buffer (1/4 volume of 4X Laemmli Sample Buffer) was added to each protein extract (50 μg/lane of gel) and the samples were boiled for 5 minutes. The samples were fractionated using 10% SDS-PAGE at 100 volts for 60 minutes. For transfer, filter paper, gels, nitrocellulose membranes were presoaked in cold transfer buffer prior to assembling a sandwich transfer and being loaded into the tank. Proteins

were transferred to nitrocellulose membrane using a transfer tank (100 V/60 minutes), on top of a stir plate. The transfer tank was cooled during transfer with ice and a stir bar was placed inside for a continuous stir. The nitrocellulose membrane was blocked with 5% milk (in TBS-T) and rocked at room temperature for 1 hour with gentle agitation. The membrane was washed with Tris-Buffered Saline-Tween (TBS-T) for 15 minutes and 5 minutes twice. Nitrocellulose membranes were probed with primary antibody (anti- iNOS, anti-COX-2, and anti-β- actin) (1:1000) and dispersed in a 0.5% Bovine Serum Albumin (BSA) solution then rocked at 4°C overnight. The membranes were washed as described above. Secondary antibody (anti-rabbit, IgG, 1:2000) was used to detect primary antibodies listed above. The membrane was washed as previously described. The membrane was exposed to ECL detection reagents for one minute and exposed to film. The film was developed manually (Appendix A).

Statistical Analysis

Three distinct experimental trials were conducted to evaluate the effect of swine management programs on the large airways of the pig. Statistical models employed for each trial are described below.

Methods for Trial I. All data were analyzed utilizing the GLM procedure (SAS Inst. Inc., Cary, NC). The breed of pig served as the main effect and the statistical model included breed only. Recorded airway measurements were analyzed by a one-way analysis of variance (ANOVA) at a p-value of 0.05 to detect significant difference between means followed by lsmeans PDiff Option as applicable to compare breed type. To determine significant difference between the means of tracheal epithelial protein level within each breed, a one-way ANOVA was employed at a p-value of 0.05.

Methods for Trial II. All data were analyzed utilizing the GLM procedure (SAS inst. Inc., Cary, NC). The pigs served as the experiment unit. The statistical model included housing only. Airway metrics were analyzed by a one-way ANOVA at a p-value of 0.05 to detect significant difference between means followed by lsmeans PDiff Option as applicable to compare all parameters measured within the indoor and outdoor environments. To determine significant difference between the means of tracheal epithelial protein level within each management style, a one-way ANOVA was applied at a p-value of 0.05.

Methods for Trial III. All data were analyzed utilizing the GLM procedure (SAS inst. Inc., Cary, NC). Due to the mixing of pigs in pens after weaning, the pens served as the experimental units. The statistical model included housing, pen and pen by housing interaction. Airway metrics were analyzed by a two-way ANOVA at a p-value of 0.05 to detect significant difference between means followed by lsmeans PDiff Option as applicable to compare all groups. Repeated body weight measurement overtime was performed to evaluate body weight variation within each management style. A correlation analysis was used to determine a possible association between body weight and airway size.

CHAPTER 4

Results

Analysis of Airway Measurements

The impact of indoor and outdoor swine production styles, on airway morphology was evaluated. Previous research has associated respiratory complications in individuals who work within confinement animal feeding operations. These respiratory complications include asthma and chronic bronchitis; as both diseases do cause morphological changes to occur within the airway of humans. It is essential to know if there is a real difference found between the two environments and the possible impact on the airway. In Trial I tracheal diameter and lumen of indoor-raised animals with different genetic backgrounds were observed. Results indicated that there were no differences found among the breed types (Fig. 4.1.A,B).

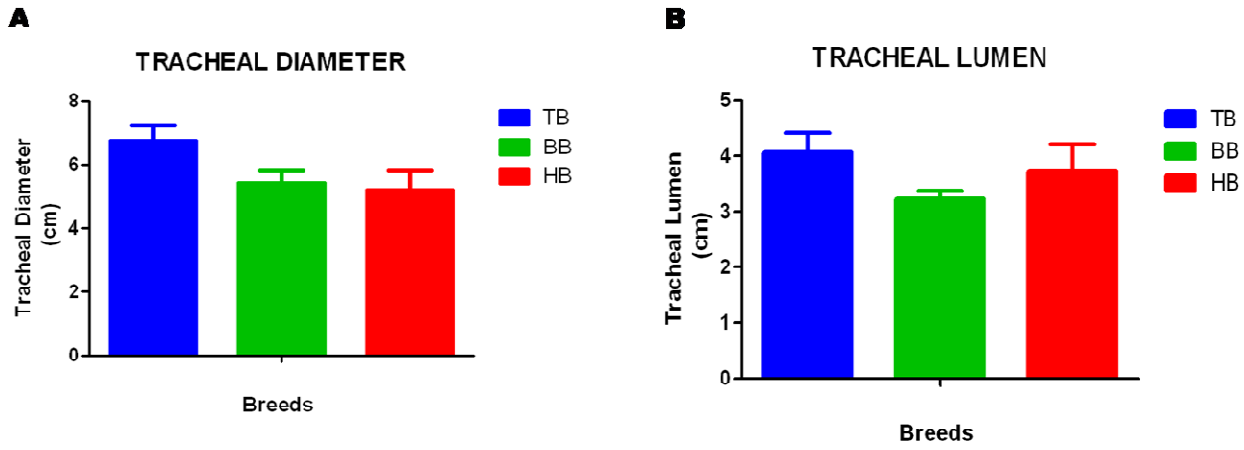


Figure 4.1. Trial I, Tracheal diameter and lumen amongst genetically distinct pigs reared indoors. Airway measurements among breed type were analyzed. Tamworth X Berkshire (TB, n = 5), Berkshire X Berkshire (BB, n = 5) and Hertford X Berkshire (HB, n = 5) pigs were used.

Figure 4.1. (*cont).* Tracheal diameter ($p = 0.1081$) and tracheal lumen ($p = 0.1962$) were not influenced by breed type. Data are shown as means ± SEM.

A second experimental trial was performed to evaluate animals reared outdoors to compare to indoor animal data from Trial I. A one-way ANOVA was preformed with a post-test of lsmean pdiff option as applicable. Tracheal diameter was greater for Trial I (indoor) than Trial II (outdoor), p = 0.0014. The lumen diameter was measured also showed a significance difference for animals reared indoors compared to outdoors (p-value = 0.0001). Figure 4.2 displays the tracheal diameter (figure 4.2.A) and tracheal lumen (figure 4.2.B) data between the two swine production operations.

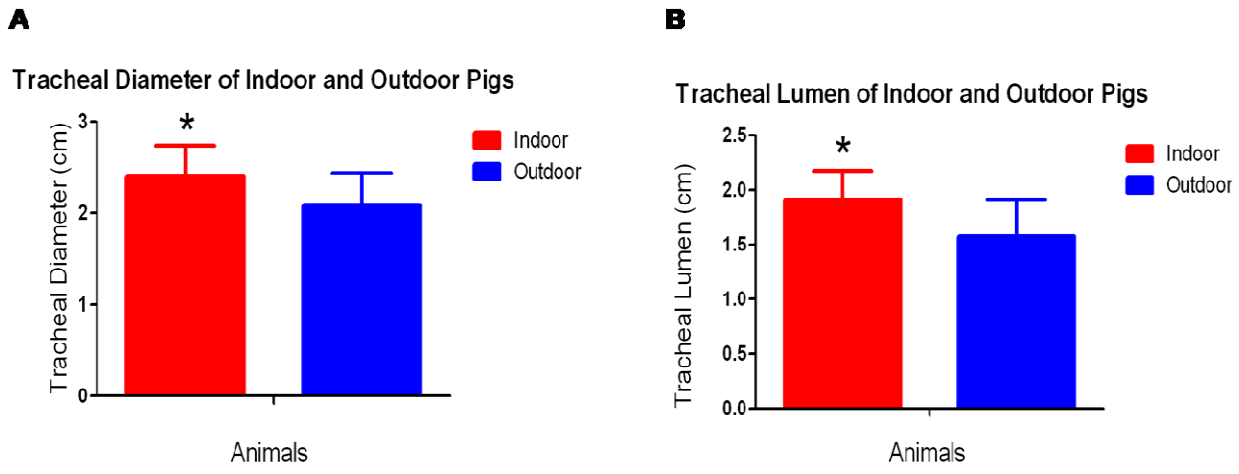


Figure 4.2. Trial II, Tracheal diameter and tracheal lumen among pigs reared indoors (Trial I) and outdoors (Trial II). There was a significant difference found, with greater tracheal diameter and lumen, for indoor (n=14) and the outdoor animals (n = 14) versus outdoor animals. P = 0.0001and 0.0014, respectively. Data are shown as means ± SEM.

After trial I and II, Trial III was performed to compare indoor and outdoor housing types at the same time. Animals were housed in pen with three rooms (indoor) or in three pastures (outdoor) with eight animals per room or pasture. As in Trial I and II, Trial III airway metrics were collected. There were no differences noted for tracheal diameter or lumen ($p > 0.05$). (Table 4.1)

Table 4.1

Airway Measurements, Tracheal Diameter and Lumen Trial III

1. Tracheal Diameter

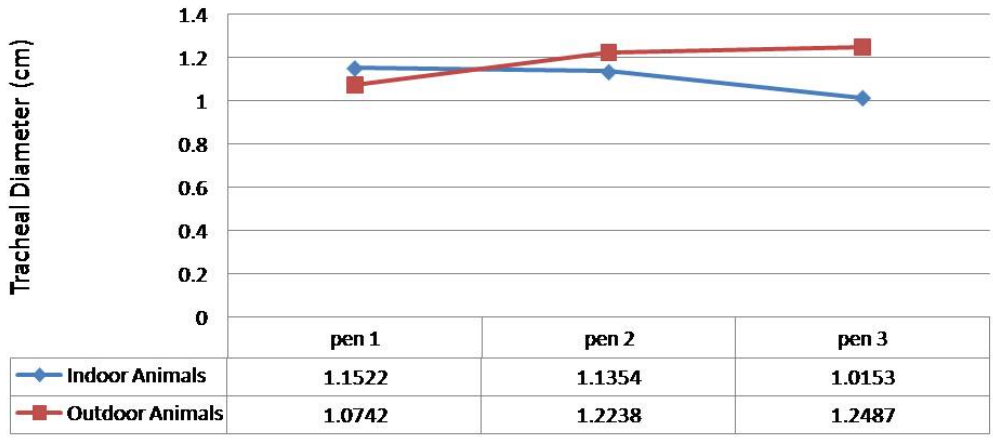
Source	DF	Type I SS	Mean Square	F Value	Pr > F
pen	2	0.07875000	0.03937500	0.99	0.3795
housing	1	0.05333333	0.05333333	1.34	0.2531
pen*housing	2	0.20041667	0.10020833	2.53	0.0924
bodyweight	1	0.00039017	0.00039017	0.01	0.9215

2. Tracheal Lumen

Source	DF	Type I SS	Mean Square	F Value	Pr > F
pen	2	0.08791667	0.04395833	1.60	0.2149
housing	1	0.03000000	0.03000000	1.09	0.3027
pen*housing	2	0.19625000	0.09812500	3.56	0.0374
bodyweight	1	0.03357637	0.03357637	1.22	0.2759

Figure 4.3 displays Trial III two-way ANOVA results of the tracheal diameter and lumen for all animals in the two environments. The mean of each treatment group was used to plot the data presented. As noted in Table 4.1 there was a significant pen by housing interaction for average tracheal lumen. This interaction suggested that there may be a confounding factor contributing to the size of the airways. Bodyweight was included into the statistical model as a covariate and was shown not to be significant (Table 4.1). Therefore, results illustrated no correlation between the size of the animal and the size of the airway for this study.

A



B

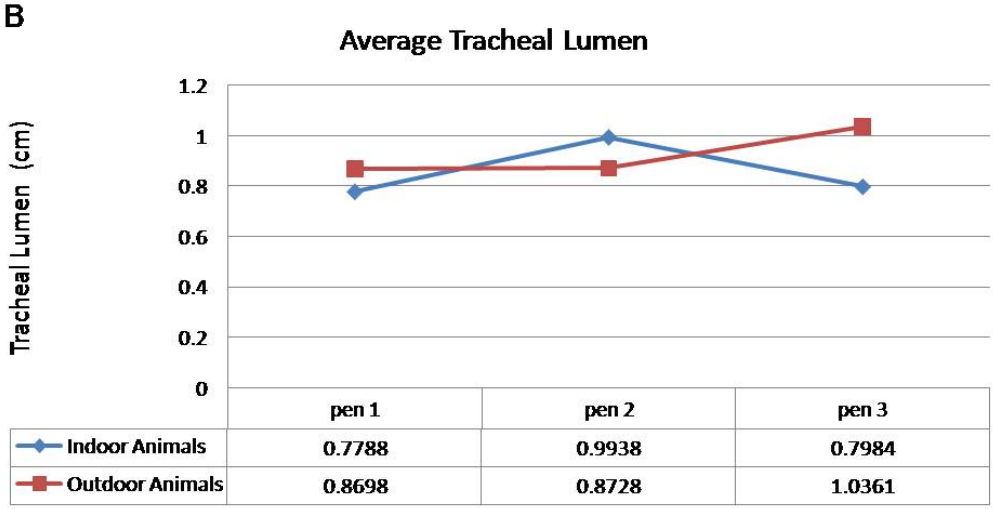


Figure 4.3. Trial III, Average tracheal diameter and lumen of indoor versus outdoor animals. There was no real difference found in any of the main effects and interaction for tracheal diameter, panel A. However, as depicted in Figure 4.3B, there is a pen by housing interaction for lumen diameter ($p = 0.0374$).

Bodyweight Variation in Pigs Reared Outdoors

Trial III was conducted in April 2011 to May 2011 and within this two month period, bodyweights of all pigs were recorded. Pigs were fed daily on a normal grower diet. Outdoor animals had the liberty to partake in more activities since they had freedom to move about within the pasture. Conversely, the indoor animals were limited by pen space and did not have much exercise. The average bodyweight within each pen/pasture are represented by in Figure 4.4. Results display a higher variation of bodyweights amongst the outdoor animals when compared to the indoor animals.

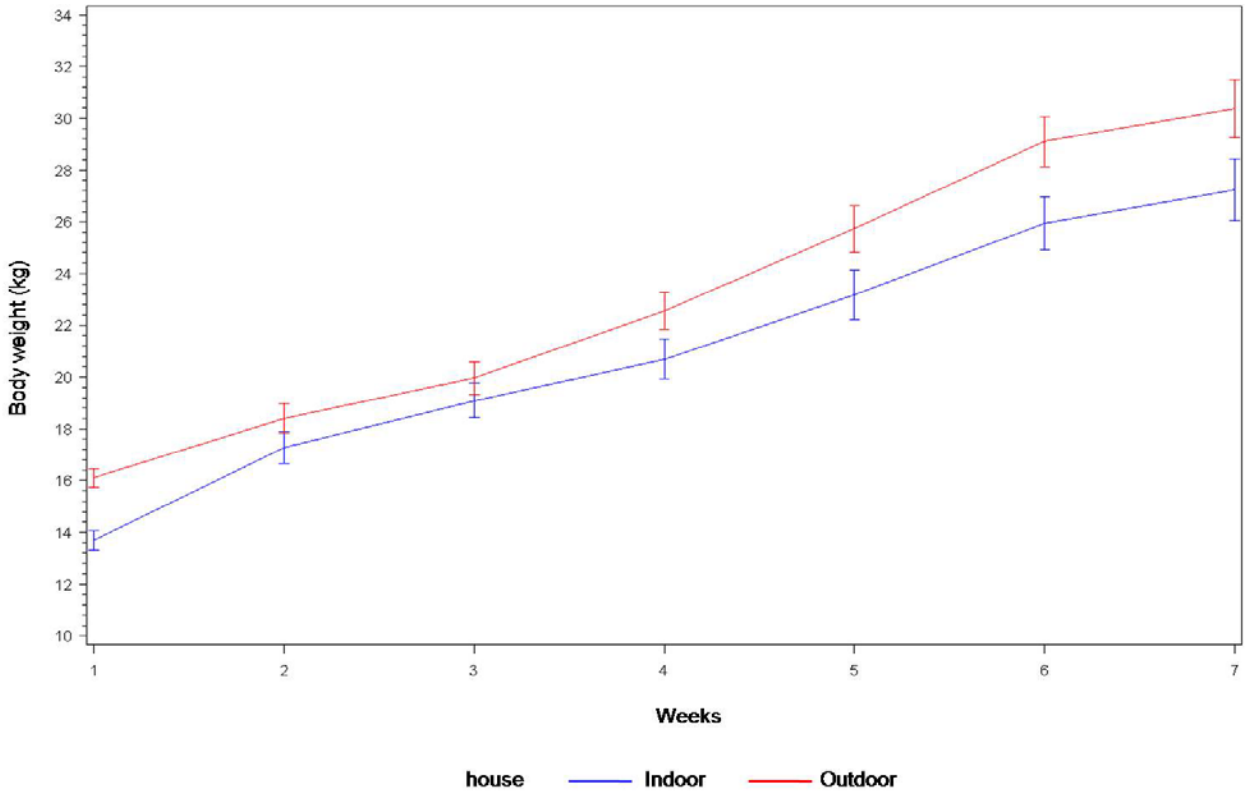


Figure 4.4. Trial III, Body weight repeated measurement overtime. Confinement animals are represented in blue and the Pasture-based pigs are represented as red. Weekly bodyweights were recorded in kilograms and the average bodyweight within each housing was plotted. Outdoor animals were shown to have a larger variation in bodyweights. Data are plotted as means ± SEM.

Basal Protein Levels of Porcine Tracheal Epithelial (PTE) Tissue

In vivo basal protein levels of tracheal epithelium from pigs were evaluated for breed (Fig. 4.5A) and housing (Fig. 4.5 B,C, D) effects. Bradford assay was performed for all Trials. Protein levels were quantitated and subjected to a one-way ANOVA. In Trial I there was no effect of breed type. When comparing Trial I and Trial II, there was a significant difference at a p-value of 0.0001. However, for Trial III in pigs raised via indoor versus outdoor systems simultaneously for a shorter time; no effect was found ($p > 0.05$) Figure 4.5 shows Trials I, II and III results. Figure 4.5D represents strictly management styles being evaluated. Basal protein levels from all animals were analyzed by housing (indoor vs outdoor) effect only. Results displayed a significant difference at a p-value of 0.0156.

Differential Airway Proteomes in Porcine Airway Epithelia

To determine differentially expressed proteins in the airways of pigs reared indoor and outdoor, two dimensional gel electrophoresis analyses were performed. Results revealed a difference between protein spot presence and abundance in samples stemming from outdoor and indoor animals. The tracheal proteomes of outdoor (approximately 30 protein spots) animals had a wide variety of proteins being expressed compared to indoor (approximately 23 protein spots) animals. Two-dimensional gels containing proteins from indoor animals had fewer spots compared to outdoor animals; however the spots were more intense for indoor animals, indicative of greater individual protein abundance (Figure 4.6).

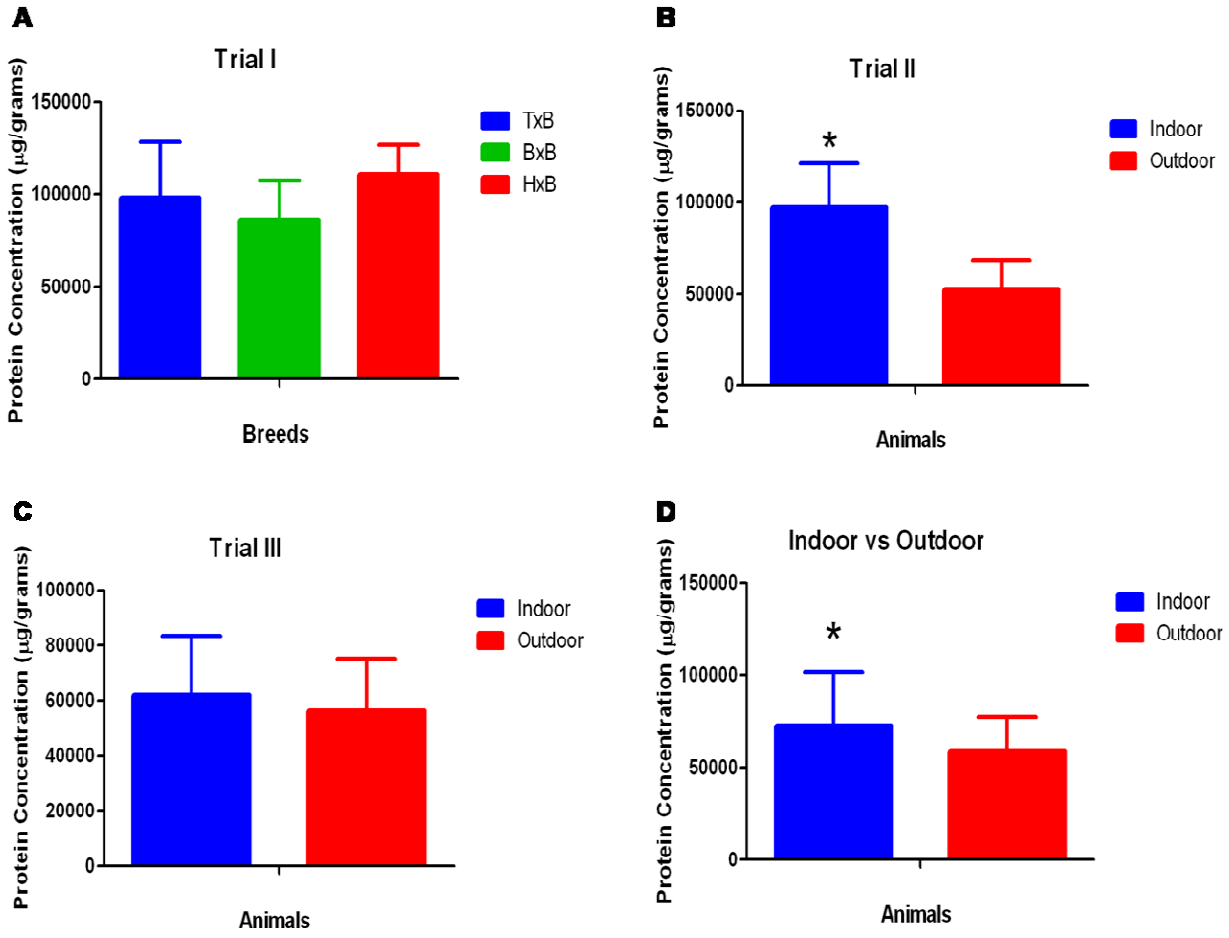


Figure 4.5. Protein concentrations in tracheal samples. A one-way ANOVA was performed to evaluate differences in basal level protein concentrations from tracheal epithelia. A, Trial I basal protein levels among breeds; B, Basal protein levels from indoor (Trial I) and outdoor (Trial II) animals; and D, Basal protein levels among all animals (Trial I, II, and III) grouped by management style. *, denotes significance: panel B, Indoor (Trial I) vs Outdoor (Trial II), p-value < 0.05; panel D, all Trials, Indoor (n = 38) vs Outdoor (n = 36), p-value = 0.0156. Data are shown as means ± SEM.

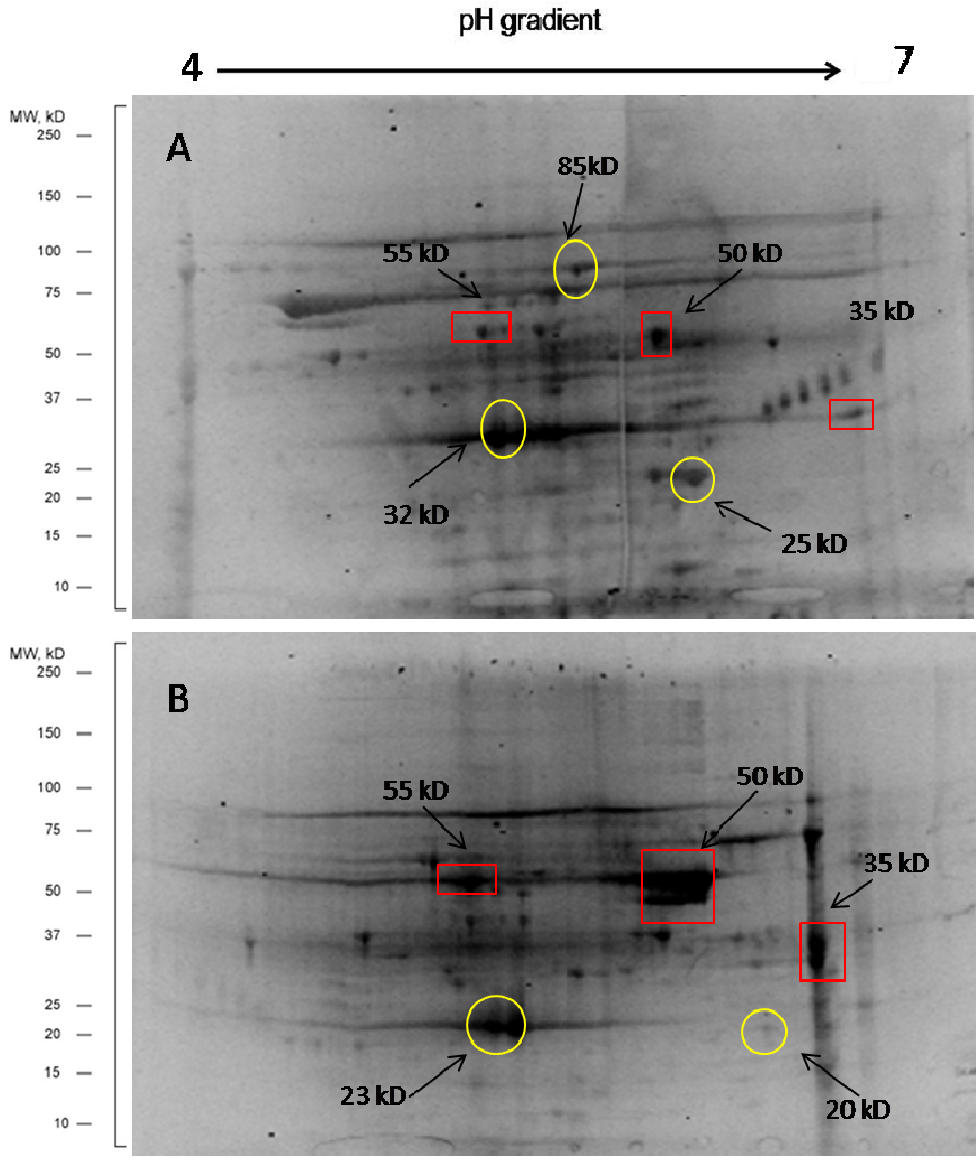


Figure 4.6. Distinct proteomes in the airways of animals reared indoors and outdoors. Two-dimensional gel analysis of airway proteome samples from Trial III, outdoor (A, n = 4, pooled) and Trial III, indoor (B, n = 4, pooled) animals reveal that there is variation between the epithelia of animals reared in the two management styles. The circles indicate similar spots and the boxes represent the different spots found within each housing environment. These gels are representative of 12 gels.

Dust extract (DE) -induced expression of iNOS and COX-2 in indoor PTE cells *in vitro*

Porcine Tracheal Epithelial (PTE) tissues were dust-stimulated *in vitro* to characterize SCF-mediated modulation of airway epithelial proteomes to provide insight for understanding SCF-mediated airway inflammation and oxidant stress. Previously isolated proteins (~60 μg/lane) were fractionated by 10% SDS-PAGE analysis and subjected to western blot analysis. Figure 4.7 shows representative western blots from porcine airway epithelial cells stimulated with DE (n = 3 sets of blots). The iNOS and the COX-2 proteins were detected at each DE concentration (1-7.5%). Results indicate that DE modulates the expression of iNOS and COX-2 proteins in PTE cells in vitro.

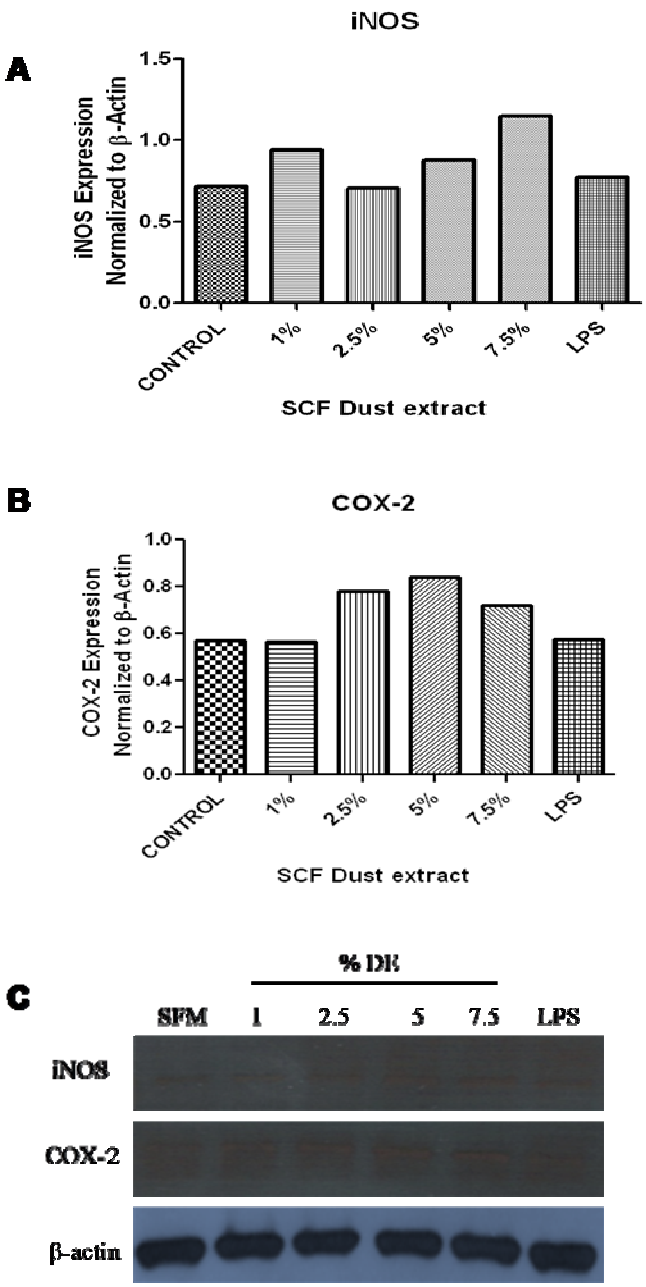


Figure 4.7. Expression of iNOS and COX-2 proteins in porcine tracheal epithelial (PTE) cells *in vitro*. Densitometric (A, B) and western blot analysis (C) of PTE cells treated with swine facility dust extract. β-actin was used as a control, (n=3); SFM = Serum Free Media. Blots are representative of three blots.

Chapter 5

Discussion and Summary

North Carolina is the second leading state of pig and pork production in the United States. Many swine farmers converted from outdoor production styles to indoor production styles to produce greater amounts of pork. Of the two primary types of porcine operations found in NC, confinement housing is typically implicated for its impact human respiratory health. A vast body of literature exists that reports the deleterious effects of reduced air quality in SCF on the respiratory health of humans. However, to date, there have been little to no studies that evaluate specifically the impact of types of operation (indoor versus outdoor) on airway morphology and function of pigs. In the present study, three experimental trials were conducted to evaluate the morphological, physiological and proteomic differences of the airways of pigs with varied genetic backgrounds reared in indoor or outdoor production settings. It was determined that the management environment may cause subtle, but distinct, differences in the airways of animals reared within them.

The trachea, also known as the windpipe, is a dynamic organ that has the flexibility to adapt and respond to changing respiratory needs. In Trial I, airway morphologies of tracheal regions were determined by airway measurements. No real difference was found when using genetic background as the main effect. In Trial II, only outdoor pigs were used but comparisons were made with indoor pigs from Trial I. There were significant differences in tracheal diameter found between Trial I (indoor) and Trial II (outdoor) pigs. Indoor animals were shown to have a larger tracheal diameter and lumen than the outdoor animals. However, this result cannot be identified as normal or abnormal. Human studies have noted that inflammation is most pronounced in subjects with no prior exposure to this environment (Palmberg et al. 2002). In addition, the indoor animals were five to six months old at harvest and had spent their entire

lifetime indoors, perhaps generating a level of tolerance to the confined environment. Trial III tested main effects of body weight, pen and housing environment and the interaction. There was no effect of environment for the tracheal and lumen diameter but there was an interaction effect for pen by housing; indicating that there may be a potential effect caused by pens within a management style. It was initially suspected that bodyweight could influence trachea or airway size. However, according to this study, Trial III body weight of pigs at 7 weeks of age did not influence the size of the airway. Other factors, such as age of animals, may have caused the pen and housing interaction to be significant. In previous reports, airway reactivity had been observed in healthy young rats of 6 weeks old compared to 21 week old rats. Results suggested that young rats are more responsive to cholinergic stimulation *in vivo*, which appeared to be at least in part due to greater cholinergic responsiveness of the airway smooth muscle (Lee, Lim et al. 2007). There were also human studies in which infant, young and old subjects were exposed to methacholine. The infants and young subjects responded more intensely (Montgomery and Tepper 1990). However, it is reasonable to accept that the anatomical airway features of young animals, whether they are pigs, rats or humans, are smaller and therefore potentially more reactive than adult airways. This data suggests the factor of age can influence how animals respond to different environmental exposures, even though the pigs in Trial I and II were of similar. Genetic variation may also explain differences observed in airway measurements. Although Trial I tested breed type, the number of experimental units may need to be increased to illustrate a significant difference. It is possible that genetic background can mediate the anatomical size of the airway. The pigs used in Trial III were still at a young age and may have not been yet influenced significantly by their environmental setting.

In Trial III, body weight was measured repeatedly over seven weeks. It was found that outdoor animals had a larger variation of body weights than the indoor animals. This can be due to many factors such as social behavior, climate, genetics, nutrition and housing. Previous studies have shown that animals reared outdoor had higher average daily gain than those reared indoors (Gentry, McGlone et al. 2002). The variation in body weights can be due to stress. Rutherford et al. (2006) conducted a study of juvenile male pigs exposed to social and environmental stress routines. Over the stressor period, weight gain was significantly reduced in stress treatment pigs compared to control pigs (Rutherford et al., 2006). Trial III was conducted in April 2011 to May 2011, in which during this time the outdoor temperatures remained at 26.7 to 32.2°C. Pigs more susceptible to temperature stress than others many have resulted in the variation seen for outdoor pigs. The litter size or the number of pigs within each pen/pasture can make a difference in feed consumption. In the beginning of the Trial III, outdoor animals were only receiving 8.16 kgs of feed. After the first week feed was increased to 10.89 kgs a day. The outdoor animals had eight pigs per pasture area, tended to eat more and had additional outdoor feed possibilities. However, the indoor animals were given 8.16 kgs of feed but there were only four animals per feeder and continued to have less variation in body weights. It is likely that management styles can impact on the respiratory health of pigs, however, the complete details of how this occurs needs additional study.

Protein levels were determined in all experiments. There were no apparent differences in protein concentrations among animals reared indoors and outdoors. However, when the samples were subjected to 2D gel analysis, distinctive proteomic expression patterns in the airways of young pigs reared in the two management operations for seven weeks was observed. This data represents an important finding because it suggests that while animals reared indoors are

generally asympotomatic compared to than their human counterparts, there are subcellular mechanisms at work which may provide more information.

An *in vitro* model was developed to evaluated cellular function for pigs using SCF DE. It is well established that SCF dust exposure stimulates and contributes to chronic inflammatory in the airways of humans (Mathisen et al. 2004; Vogelzang et al. 2000). Due to the physiological and proteomic similarities of pigs and humans, pigs may suffer from similar respiratory symptoms as their human counterparts following SCF DE (Gerald et al, 2010). Results from this preliminary study have shown that pro inflammatory proteins, cyclooxygenase (COX-2) and inducible NOS (iNOS) are up-regulated and have experienced a partial DE dose-dependent response when PTE are exposed to SCF dust for 8 hours. This outcome was to be expected. Gerald et al. (2010) conducted an experiment exposing SCF DE to Porcine Tracheal epithelial cells and display evident expression of the iNOS protein was at each concentration dust gradient level of 0.1%, 0.5%, 1% , 3% and 5% (0 – 5%) DE. COX-2 was also detected at each concentration level (0-5%) in a concentration-dependent manner. These results are important because (COX-2) is a known inducible type of enzyme that participates in promoting inflammatory reactions and is associated with a range of inflammatory diseases such as arthritis and asthma in humans. It is also responsible for converting arachidonic acid into several types of inflammatory mediators including prostaglandins and leukotrienes (Szczeklik et al. 2002; Gylifors et al. 2007); and both of these classes of molecules have been associated with upper and lower respiratory diseases. In addition, iNOS is induced by inflammatory cytokines. In asthmatics, iNOS has been shown to be essential in airway inflammation and remodeling (Prado et al. 2006).

CHAPTER 6

Conclusion

Commercial indoor operations and pasture-based outdoor operations are two common management programs used in the swine industry. Most pork produced in the United States come from animal raised indoors. However, literature has shown that individuals working SCF may develop respiratory illnesses such as chronic bronchitis and asthma-like syndrome. Commercial operations have been shown to elicit airway inflammatory responses due to the accumulation of dust, dried manure and gases that can contribute to poor air quality. The current study provides insight for understanding the impact of swine management environment on the airways of pigs.

This study provides a comparison of the porcine management styles and essentially its impact on porcine airway epithelia tissue. Our results indicated that when using breed as a main effect there is no difference found when observing tracheal diameter, tracheal lumen and basal tracheal epithelial protein levels. However, in trial II, when exclusively evaluating animals based on whether they were reared indoors or outdoors there was a significant difference in airway metrics. The indoor animals from Trial I was found to have a larger tracheal diameter and lumen compared to animals that were reared outdoors in trial II. The indoor animals from Trial I were also shown to have increase levels of tracheal epithelial protein when compared with outdoor animals in trial II. This data gives evidence that there may be a morphological and proteomic difference found between the two types of hog operations. Trial III showed no significant differences in tracheal diameter and lumen as well as tracheal epithelial protein basal levels. However, data did show an interaction with pen by housing in the average tracheal lumen model. Bodyweight was placed with the statistical model and was shown to not be a confounding factor.

This suggests that other possible unknown confounding factors exist, in which was not identified in this study such as breed, age and season.

The proteomic analysis portion should be further investigated. In this study only animals from the two management operations were tested. Further explorations involving genetic background will be necessary to really understand the possible effects exerted by breed differences. Larger cohort studies are needed with more sires and lines within the breeds for many year and multiple repetitions to obtain the significance of a population study. Protein identification needs to be taken into consideration. Identifying the differential proteins could give us insight to biological mechanisms that occur in the animals and humans exposed to these types of environments.

In vitro fractions we tested only for COX-2 and iNOS as pro-inflammatory proteins. More proteins should be evaluated and addition animal and sample numbers should be included; along with different ages, weights and exposure time. Further proteomic investigation may include the evaluation of oxidative, mucin, bacterial and other proteins that may be found in the airway epithelium.

Although promising results were generated from this study there were several experimental challenges. Often times it was difficult to obtain strictly indoor and outdoor animals from North Carolina A & T State University Swine Farm Research Unit. It was a challenge to find abattoirs to purchase and or process piglets less than 120 pounds. It was too difficult to establish sterile culture of PTE cells from animals reared outdoors. Due to the animals exposure to the outdoor environment the inability to establish a sterile cell culture prevented in vitro studies on PTE cells to evaluate cell function.

Future directions may include more studies to evaluate those confounding factors like age, breed and body weight over time. These variables can be analyzed and compared to the size of the airway of young and adult pigs. When comparing trial I indoor animals to Trial II outdoor pigs, there was a difference displayed. More studies pinpointing the cause of increased size of airway morphology as well as protein levels may be need to be conducted.

In conclusion, animals reared indoors may have distinct airway morphologies and proteomic profiles compared to those reared outdoors, which may, in part, explain their ability to live within confinement houses without any apparent complications.

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Appendix A

Protocols

Protocol #1: Protein Isolation

1. **Preparing RIPA (1x) Lysis Buffer**
   RIPA is originally a (10x) solution that will need to be diluted to (1x) in this procedure. In a 125ml glass bottle with a screw on top, add 90mLs of deonized water and 10mls of RIPA 10x Buffer. Place top onto bottle. Invert the mixture inside the bottle for further mixing about 10 times. Place on ice when in use and stored at 4°C.

2. **Make Tablet Stock (Complete Protease Inhibitor Cocktail tablet)**
   In a 15 ml cryogenic tube add 5mls of RIPA (1x) Lysis Buffer
   Add 1 tablet of complete Protease Inhibitor Cocktail
   Invert tube and make sure the tablet is completely dissolved.
   Place on ice when in use and stored at -20°C

3. **Remove Swine trachea from the -80°C freezer and place sample on ice for 15 minutes.** This should be enough time for it to partially thaw out. We don't want it completely thaw. Try to keep the sample cold.

4. **Once tissue is partially thawed.** Cut trachea longitudinally to create a spread. Remove the epithelium layer of tissue from the inside of the trachea. Try not to include the cartilage but if so it is okay.

5. **Place epithelium lining inside a 15ml cryogenic tube and place on ice.** Obtain a weigh boat and a spatula. This will assist you with finding out the weight of the tissue. Turn the power on (Scale: Mettler Toledo; SNR: 11 20 122 771) Place the weigh boat onto the scale and press tare to cancel out its weight. Place the epithelium lining onto the weigh boat and record the weight in your lab note book. (Average weight used is 1.2g)

6. **Depending on weight make extraction buffer 0.5g/1ml solution Extraction buffer**

   In 50ml cryogenic tube, add 2200ul of RIPA (1x) Buffer, 25ul of protease inhibitor P8340 (stored at -20°C), 25ul of phosphatase inhibitor P2850 (stored at 4°C), and 250ul of Tablet Stock (stored at -20°C). This makes up a 2.5mls of extraction buffer.
   In 50ml cryogenic tube, add 2640ul of RIPA (1x) Buffer, 30ul of protease inhibitor P8340, 30ul of phosphatase inhibitor P2850, and 300ul of Tablet Stock. This makes up a 3.0mls of extraction buffer.

7. **Add the weighed epithelium tissue into the extraction buffer tube and homogenize sample.**
   Sample will be ground for 10 seconds 3 times, in between the 10 seconds let sample sit on ice for one minute.
   Spin homogenized sample for 30 minutes, 4°C at 6000 RPM [Centrifuge used: IEC Centra – 7R refrigerated centrifuge] *SN: Make sure samples are balanced in the centrifuged. At this point you should be using the 50 ml cryogenic tubes

8. **Supernatant should be collected and placed in eppendorf tube.** * Be sure not to collect any of the residual that may surface to the top of the sample.

9. **Spin 30 minutes,** 4°C at 10,000 RPM [Centrifuge used: Thermo Electron Corporation Sorvall RC 6 Plus] SN:*Make sure samples are balanced in the centrifuged. At this point you should be using the 1.5ml cryogenic tubes.

10. **Supernatant should be collect and placed in a new eppendorf tube.** *Be sure not to collect any of the residual that may surface to the top of the sample.

11. **Spin 30 minutes, 4°C at 10,000 RPM** [Centrifuge used: Thermo Electron Corporation Sorvall RC 6 Plus] SN:*Make sure samples are balanced in the centrifuged. At this point you should be using the 1.5ml cryogenic tubes.

12. **Supernatant should be collect and transferred to a new eppendorf tube**. Place on ice immediately. *Be sure not to collect any of the residual that may surface to the top of the sample.

    Sample should be ready for Bradford Assay!

Protocol #2: Bradford Assay

96 well plate was used

1. Use two (2) sets of BSA standards (800, 400, 200, 100, 50, 0 mg/ml) stored at 4oC.
2. Dilute the Bradford Reagent Assay Dye (stored at 4oC) 1:4 in dH20.
3. Prepare a 1:100, 1:200 and 1:300 dilution of each unknown sample in triplicate.
4. Add 10 µl BSA standards, 10 µl unknown sample dilutions (in triplicate).
5. Add 190 µl diluted Bradford Assay Dye to each sample well.
6. Read plate using plate reader.
    a. Turn on VERSAmax Microplate Reader and put plate in drawer. Press "Drawer" button to open/close drawer.
    b. Click "SoftMax Pro v5" icon.
    c. Go to "Protocols" drop-down menu.
    d. Go to "Protein Quant."
    e. Go to "Bradford."
    f. Click "Template" to enter plate/sample information (i.e., Standard ID, Unknown Sample ID…).
    g. Click "Read."
    h. When finished, save and print results & standard curve
    i. MAKE SURE DRAWER IS CLOSED & COVER INSTRUMENT.

Protocol #3: Two Dimensional Gel Electrophoresis and Trypsin Digestion for Mass Spectrometry

Dos and Don'ts of Proteomics Sample Preparation:

- **NEVER TOUCH PROTEOMICS GELS OR SUPPLIES WITH BARE HANDS!**

- PMSF (phenylmethylsulfonyl fluoride) is a chemical that inhibits trypsin (the enzyme used to digest proteins for Mass Spectrometry). **NO PMSF** should be used, this will ruin your results.

- **NO Cell Signaling Lysis Buffer™**; **NO polymers** such as polyethylene glycol (PEG) should be used with in preparation of protein samples. However, if cell signaling lysis buffer is used in previously prepared samples (which contains ethylene glycol tetraacetic acid (EGTA)), the sample **must** be filtered through a YM10 column (Millipore) to remove the polymer (size range ~4-8 kDa).

- **RIPA lysis buffer** can be used because it is a "Tris" based lysis buffer. You may add protease inhibitors to this basic lysis buffer to make an extraction buffer.

Pooling protein samples:

Starting from the end of the Bradford Assay Protein Concentration you are to divide the amount of samples you are using by the total amount of protein you will use. For example, (in my case) it would be 4 samples, and divide that into 500 µg, the total amount of protein that I run with a 2D gel. **We have optimized 500 µg/IPG strip** however; the protein range for IPG strips (Bio-Rad) used in our lab is 200-1000 µg.

Ex: 500 µg/4 samples = 125 µg/sample

This means that I will be pipet 125 ug from each sample into a separate tube for a total of 500 µg.

To get 125 µg of protein, I will be dividing 125ug by my stock protein concentration (e.g., 37.5 µg/µL) determined by Bradford Assay or Nanodrop method.

Ex: 125 µg/37.5 µg/µL = 3.3 µL of my stock protein extraction sample will be added to the tube for IPG focusing. I will repeat this step for each sample and my final protein amount will be 500 µg.

Tips to Avoid Keratin Contamination

- **NEVER TOUCH PROTEOMICS GELS OR SUPPLIES WITH BARE HANDS!**
- Before starting any experiment that involves proteomics, please remember to wash your hands and gloves once they are placed on. This is to eliminate or reduce all possible contaminations.
- From now on ONLY sterile tips and tubes (low binding proteins tubes) should be used to run this experiment.
- We will use glass dishes to maintain the gels prepared and be sure to have limited contact with the gels. (**DO NOT TOUCH THE GEL**) All dishes must be washed with soap and water, left out to dry and rinsed off with deionized water before use.

Rehydration Step (day 1):

1. Thaw IPG Ready Strips on lab bench for no longer than 15-20 minutes.
2. In a small low binding protein tube or sterile tube add your samples and rehydration buffer, both solution should total up to 185ul, which is the maximum amount that can be used on the IPG strip that will be used.
3. Vortex mixture for 15 seconds
4. Fill 185ul if sample mixture in the first well of the rehydration tray. *Be sure to distribute sample evenly into the middle of the well so the entire strip will be submerged into the buffer.
5. Peel the IPG strip and place the gel side down. * No air bubbles please
6. Pipette 2mls of mineral oil on top of the strip
7. Cover the rehydration tray with plastic cover and wrap.
8. Refrigerate overnight

Isoelectric Focusing Phase (day 2):

1. Remove rehydration tray from the refrigerator
2. Place pre-cut wick on top of each electrode that will be occupied by a IPG strip on the focus tray
3. Pipette 8.0ul of nanopure water over each wick
4. Drain the IPG strip (from rehydration tray) from the mineral oil; placing its tip onto the kem wipe *DO NOT Touch the Gel Side of the strip
5. Place gel side down in focus tray directly on top of the wicks
6. Transfer 2mls of mineral oil on top of strip
7. Place focus tray with IPG strips in to the focus box (matching positive to positive and negative to negative)
8. Set setting of IEF Instrument (~6 hour run)

IEF Settings (11cm)

1. Preset Method
2. Linear
3. Highlight "11" (No Rehydration)

*DO NOT touch temperature

4. Next Screen

S1 250V 15
S2 Hr:Min v hours
S2 8,000 2:30

_____

S3 Hrs:Min vhours
S3 8,000 20,000
S4 500v/Hold? Yes

_____

Next
Method linear
Limit/ Gel 50 uA
gels: 1 (this number will change depending on the number of strips being used)
Press start (it will read 'Run in Progress')

When finished:

1. Stop it, Cut it off and take focus tray out
2. Obtain clean rehydration tray
3. Take strip out of focus tray and drain off the mineral oil, using kem wipes
4. Place strips inside rehydration tray; same orientation (+3 to -10)
5. Place cover on rehydration tray and wrap with plastic wrap; store in -80°C overnight

Running the Gel (day 3):

1. Remove tray from the freezer and let strips sit at room temperature; no longer than 20 minutes.
2. During this time prepare both equilibrium buffers (buffer I and II)
3. Pipette 4mls of buffer I onto each strip and place on rocker for 10 minutes.
4. At the 10 minute mark, drain off buffer one
5. Pipette 4mls of buffer II onto each strip and place on rocker for 10 minutes.
6. At the 10 minute mark, drain off buffer two.
7. Remove pre-cast gel from the refrigerator
8. Take off green comb and white strip at the bottom of the case.
9. Use the kem wipes to dry out the well of the gel.
10. Heat up the agarose gel in the microwave for about 45 seconds, this will liquefy the agarose.
11. Make up a TGS(1x) solution in a 1000ml graduated cylinder

Ex/ 100mls (TGS) + 900mls (deionized water)

12. Pour 50mls TGS(1x) solution into a 50ml graduated cylinder
13. Dip entire strip into the 50ml TGS(1x) solution at least 5 times or just enough to coat the whole strip
14. Fill well with heated agarose gel
15. Place strip into the pre-cast gel well. *The gel side of the strip should be facing towards the backside of the gel case.
16. Use pipette tip to make sure strip is completely inside the well *no air bubbles please
17. Let sit for at least 10 minutes
18. After the agarose gel has solidify, place gel cassette into gel box
19. Fill gel box with the TGS (1x) solution until the "Fill line" and the cup of the plate with the rest of the TGS buffer
20. Place lid and set settings: 11cm, 200V, constant, 65 minutes

When Gel Run is completed (Staining):

- *Rinse with deionized water between each step

1. Remove cassette, detaching it in half. DO NOT touch the gel and gently place it in glass dish

First Gel (running gels in parallel):

2. Fix gel in a 40% methanol, 10% Acetic Acid mixed solution for 30 minutes
3. Stain with Sypro Ruby stain reagent for at least 90 minutes (this step can be done overnight if needed)
4. Destain in a 10% methanol, 7% Acetic Acid mixed solution for 30 minutes
5. Stain with Coomassie Blue

Second Gel:

6. Fix gel in a 40% methanol, 10% Acetic Acid mixed solution for 30 minutes
7. Stain with Simply Blue *refer to simply blue protocol* – ***Title: 'Using Simply Blue Safestain'***
8. Image both Gels

*DO NOT pour or discard Sypro Ruby Stain solution; please return back into its original container for later use
*Simply blue is non toxic, which means it can be discarded down the sink.
*Coomassie Blue solution CAN NOT be discarded down the sink.

- **YOU MAY ALSO RUN ONE DIMENSIONAL GELS FOR PROTEOMICS STUDIES. SEE PROTOCOL BOOK FOR SDS-PAGE PROTOCOL (you can find this with Western Blot Protocols). Use the same precautions described here for proteomics. Run the gel and according to the specifications for 1D gels and use the SimplyBlue Staining Protocol.**

To view these images:

1. Hines Hall: Gel Imager with the option to overlay gels
2. Barnes Hall: Gel Doc Imager

After Imaging Gels:

1. Be sure to take picture
2. Store gels in deionized water at $4^{o}C$ for a short time if needed (few days)

Cutting out Protein Spots:

- It is for you to decide what spot interest you. For example, in a comparative study with control versus treated, you may want to 'pick' spots that are absent and present between the two samples. A densitometer will be needed to determine changes in spot/band intensity. Also, fluorescent labels/tags (e.g., SILAC, iTRAQ, Cy Dyes) may be incorporated for differential proteomics. The use of these methods require a fluorescent imaging system.

- Take a picture of the gels and label the spots/bands on your image, so that when collecting the spot (into low binding protein tubes) we will know where these spots came from.

- To cut bands from gels, place the gel on a clean GLASS plate (wash with soap and water, rinse with dH2O but do not dry), use a clean razor blade to cut bands from gel (try to avoid excess gel, too much gel will decrease efficiency of trypsin reaction…ONLY cut the band)

- You MUST also cut out a piece of the gel that does not contain spot, just to evaluate the background (Tyrpsin digestion will be perform on this too)

- Trypsin digestion (Reducing agent used per volume: 1 to 10 volume)

- This method should be performed the same day protein spots are collected.

Trypsin Digestion:

In Gel Digestion - *Please refer to protocol – ***Title: 'Tryptic in-gel digestion of Proteins'***

In Solution Digestion - *Please refer to protocol – ***Title: 'Tryptic in-solution digestion of Proteins'***

Send solid powder to Penn State Proteomics and Mass Spectrometry Facility (Dr. Tatiana Laremore, Director) to be evaluated

Protocol #4: Isolation and Expansion of Porcine Tracheal Epithelial (PTE)

Porcine Airway Acquistion

1. Porcine airways (trachea & portions of bronchi) were dissected from freshly killed hogs, rinsed with cold tap water, placed in plastic bags and kept on ice during transport. Airways were on ice approximately 2-3 hours.
2. Any excess connective tissue (lymph nodes, heart and lung) were removed from specimens using sterile forceps, tweezers, scalpel.
3. Trachea and bronchi were used. Waste tissues were placed in biohazard bags and stored at -20°C for EHS pick-up and disposal.

Tissue Preparation and Dissociation

4. Airways were cut into smaller rings(1-2 inch = 2.5-5 cm), placed in sterile beakers with ice-cold wash media (HBSS containing antibiotics: Pen-Strep & Ampho B).
5. Airway rings were washed three times using Wash media; swirling with each new addition of media.
6. Airway rings were slit open with scalpel and cut into 1 x 2 cm segments.
7. Tracheal segments were transferred to 50 ml conical tube(s) containing 30 ml of dissociation media (DMEM with Pen-Strep & AmphoB) plus 4 ml of 10 X Protease/DNase solution. Approximate tissue to fluid ratio should be 1:9. Final volume should be 40 ml.
8. Tracheas were digested with 0.1% protease XIV and 0.001% DNase in Dissociation Media at 4°C for 24 hours with gentle agitation (on a platform rocker)

Cell Harvesting (The Next Day)

9. Followed sterile tissue culture techniques under a laminar flow hood.
10. Ended dissociation reaction by adding fetal bovine serum to a final concentration of 10% (4 ml to 40 ml) to neutralize protease. Inverted tubes to mix.
11. Dissociation reaction tube contents were poured into a sterile 150-mm tissue culture dish, one tube at a time.
12. Harvested cells by gently scraping internal surfaces of airways with a surgical scalpel blade to slough off airway cells.
13. Rinsed tissue surfaces and collection dish with sterile tissue culture grade PBS and pooled solutions containing dissociated cells into 50 ml conical tubes.
14. Centrifuge at 5000 rpm for five minutes using centrifuge.
15. Wash cells in Dissociation media (without protease/DNase), spin again, and resuspended in a small volume (3-5 ml) of Porcine Complete “Expansion” Media
16. Count cells using a hematocytometer and determine viability by trypan blue exclusion assay

a. Briefly mixed 20 ml of cell suspension with 20 ml trypan blue dye solution. Let stand 2 minutes at RT. Add 10 ml to hematocytometer via caplillary action. Counted 5 squares (four corners and center) Calculated cells per ml.

Seed Cells in Vented T-75 Flasks for Expansion (Initial Grow-Up Step)

Porcine airway epithelial cells for *in vitro* culture.

17. Adjusted volume of cells to obtain a concentration of ~500 cells/cm^2 or 3.75-5.0 x 10^4 cells /ml per flask using Porcine Complete Expansion Media
18. Added 14 ml Porcine Complete Expansion Media to each vented T-75 flask and 1 ml of cells (~500 cells/cm^2 or 3.75-5.0 x 10^4 cells).
19. Swirled gently to evenly distribute cells.
20. Checked cells under microscope to ensure they were distributed evenly.
21. Incubated cells at 37°C, 5% CO_2 with humidified air overnight.
22. Changed media. Feed cells every other day until they reach 80-90% confluency. Maintained Expansion log. Check cells daily and record confluency.
23. When cells have reached 80-90% confluency (6-8 days), harvest cells using cryopreservation protocol
24. Stored cells in liquid nitrogen as 1.0 ml (2.0-2.5 x 10^6 live cells/ml) aliquots in freezing media.

Table 1. Porcine Complete "Expansion" Media (DMEM/Ham's F12 plus supplements, High EGF)

Supplement/Additive	Stock concentration	Volume	Final Concentration
Penicillin/Steptomycin Mix, -20°C	10,000U/10 mg/ml	500 µl	100 U/0.1 mg/ml
Amphotericin B,-20°C	250 µg/ml	2 ml	1 µg/ml
Epidermal Growth Factor (EGF) -20°C	12.5 µg/ml	500 µl	12.5 ng/ml
Transferrin, -20°C	5 mg/ml	500 µl	5 µg/ml
Insulin, -20°C	5 mg/ml	500 µl	5 µg/ml
Triiodothyronine (T3), -20°C	20 µg/ml	500 µl	20 ng/ml
Fetal Bovine Serum (FBS), -70°C	100%	5 ml	1%
Retinoic Acid, -70°C	15 mg/ml	500 µl	15 pg/ml

Protocol #5: PTBE Cell Harvest & Cryopreservation

1. Aspirated media, rinse T-75 flasks with 8-10 ml HBSS buffer.
2. Added 5-8 ml of warm trypsin (thaw in 37°C water bath) to each flask. Incubated at 37°C for 5 min, rocking flask every 1-2 minutes or until most of the cells have detached.
3. Immediately added 5-10 ml of cold Trypsin Neutralization solution to each flask.
4. Transferred cell suspension to a 50 ml conical tubes.
5. Rinsed flasks with 8-10 ml cold HBSS/ flask and add to conical tubes containing cell suspensions.
6. Spun at 4000 rpm for 5 minutes at 4°C.
7. Removed supernatant by vacuum aspiration.
8. Tapped tube to remove clumps prior to adding media to cells.
9. Used ice-cold Expansion media (with high EGF, 25 ng/ml) to resuspend and wash cells. Pool into one tube.
10. Spun cells again at 4000 rpm for 5 minutes at 4°C.
11. Determine cell concentration and viability using Trypan Blue Exclusion Dye (20 µl dye + 20 µl cell suspension). Count with hemacytometer.
12. Resuspended cells to a concentration of 2.0 x 10^6-2.4 x10^6 live cells/ml in Cell freezing Solution (80% PTBE expansion media, 10% DMSO; 10 % Heat inactivated FBS).
13. Made 1 ml cell aliquots. Using 2.0 ml cryotubes with internal threading and silicon gasket.
14. Placed cryotubes inside a Styrofoam cooler; place at -80°C, overnight.
15. Transfered cells to liquid nitrogen storage the next morning.

Protocol #6: Protocol for Growing PTE Cells

NOTE: Always use sterile technique while performing tissue culture procedures. Refer to *"SOP for Sterile Technique and Tissue Culture Equipment"* document for details.

Day 1: Seeding Cells

1. Select plate type 6-well (plastic only/transwell insert-for going to air).
2. Label plate(s) with Name, Date, Plate # and donor (e.g. CM8111).

3. **Make collagen coating solution** to coat wells by diluting collagen stock in 0.02 N Glacial Acetic Acid (see pg. 4).
    a. Collagen Stock Solution: 3.13 mg/ml ≅ 3.13 μg/μl
    b. Desired final concentration = 50 μg/ml
    c. For example → 50/3.13 = 15.97 ∴use 16μl collagen stock/1 ml acetic acid.
4. Add 0.5 ml/well and swirl plate to achieve complete well coverage.
5. Let stand at RT for 1 hour.
6. Vacuum-aspirate excess collagen solution and wash wells with 1 ml PBS for 10 min at RT. Swirl to wash sides of wells.
7. Remove PBS.
8. Add 2 ml of conditioning media (DMEM) to wells and incubate @ 37°C/5% CO_2 for 15 min-1 hour while P50:50 Base Media and Porcine Complete Media are made.
9. **Preparing P50:50 Base Media (viable for 8 weeks @ 4°C)**
    i. Obtain an unvented T-75 tissue culture flask
    ii. Obtain Ham's F12 & DMEM and **mark fluid level in Ham's F12 bottle**
    iii. Pour 250 ml of Ham's F12 into flask and 250 ml of DMEM into Ham's F12 bottle up to the marking.
    iv. Add 250 ml of Ham's F12 from flask to DMEM bottle.
    v. Label accordingly with "50:50 F12:DMEM" , initials, date and refrigerate.

10. **Preparing Porcine Complete Media (viable for 6 weeks @ 4°C). Let additives thaw at RT for 15-30 min in DARK.**
    i. Get the following reagents from -20°C: FBS, Penicillin/Streptomycin Mix, Amphotericin B, EGF, Transferrin, Insulin, Nystatin & Triiodothyronine (T3)
        1. After you thaw 50 ml of FBS, aliquot the rest into 10 ml/tube at store at -20°C.
    ii. Get the following reagents from -70°C: Retinoic Acid (light sensitive)
    iii. Add all (9) components to P50:50 bottle, label bottle "**Porcine Complete**" date, initial & refrigerate. Complete Media is stable for up to 6 weeks.

11. Label two, sterile 50 ml tubes for complete media as follows: **1)** Wells and **2)** Cells
    a. Add **"2 ml/well"** in 50 ml tube (**media for Wells**) and **"1 ml/well"** in a 50 ml tube (**media for Cell resuspension & distribution** into wells)
    b. Warm both tubes in 37°C water bath/incubator for 15-30 min.
    c. **NOTE:** Warm only the amount of media required for immediate use. Do not warm entire bottles of media.
    d.
12. Vacuum-aspirate conditioning media and add 2ml Porcine Complete Media to wells (use "2ml/well tube").
13. Determine how many vials of cells you will need (2.0×10^6 Live PTE cells/vial) to seed the plates. **Generally use ~ 1×10^4 cells/well** (e.g., 3 6-well plates ≅ 18 wells = 13,888.88 cells/well).
    a. **Cells are kept in liquid nitrogen tank**.
    b. **Remember to mark log when you take cells (with an X, initials & date)**!
        i. When retrieving LN_2-frozen cells get an ice bucket with lid. Tubes have been known to burst.
        ii. In hood, partially unscrew cap to make sure no LN_2 is in the threads of the vial. **Then close vial tightly again.**
        iii. Quickly thaw cells for 1-2 min in 37°C water bath. ***Keep the O-ring above the water to reduce the risk of contamination.***
        iv. Add cells to pre-warmed media tube with "1 ml/well" volume. Pipet twice (gently) to mix.

14. Pipet 1 ml of cell suspension into each well (or transwell chamber if "Going to Air").
15. Gently swirl plates to get even cell coverage within wells. Check cells using a microscope. Incubate at 37°C/5% CO_2 over night.
16. Record seeding concentration and daily growth notes using a cell culture log. Attach to your lab notebook.

Day 2-6: Feeding Cells

1. Pre-warm (37°C water bath/incubator) 3 ml/well complete media for 15-30 minutes.
2. Vacuum-aspirate all spent media without touching the cells/cell surface.
3. Add 3 ml of media to each well (OR 2 ml to bottom + 1 ml transwell chamber if using transwells for "Going to Air").
4. Gently swirl plates and place back in incubator.
5. **Wait two (2) days before feeding cells again. Then feed them every other day until they reach 90-95% confluency; *around ~Day 5-7*** depending on initial seed concentration.
6. **Visually inspection cultures daily with natural vision and microscope for cell growth/health and possible signs of contamination.**
    a. Report any contaminations to the lab manager or Dr. Waterman.
    b. Avoid over growth of cultures, especially with primary cells because they will display contact inhibition and begin to die.

c. *Contact inhibition* is a natural process of arresting or "inhibiting" cell growth when two or more cells come into contact with each other. Cancer cells do not show contact inhibition and this is a phenotype oncologists use to distinguish between normal and cancerous cells.

7. Proceed with experiments OR "Going to Air" protocol.

Day 7-21: Going to Air

1. Vacuum-aspirate media in both chambers.
2. Add 2 ml pre-warmed media to the **bottom of well only**. Gently swirl plates and return to incubator.
3. **Cells must be fed, via the lower chamber ONLY, everyday** at this point **(even on weekends) for 14 additional days.**
   a. **NOTES FOR SUCCESSFUL ALI EXPERIMENT…**
   b. Change media at the same time every day and only warm the amount you need for the day.
   c. **If you accidentally drip media on the cell surface during media changes, gently remove it via aspiration taking care not to disturb the cell surface or mucus layer.**
   d. **Pipet slowly to avoid the formation of air bubbles. Air bubbles prevent cells from accessing media.** If they form, swirl the plate to move them to the side of the well. If that does not work, use a sterile 1ml serological pipet or Pasteur pipet to gently lift one side of the inert using the ONLY the UPPER RIM of the transwell…just enough to allow the bubble to escape. **You should never touch the transwell membrane. Ask Dr. Waterman for assistance if necessary.**
   e. As the cells differentiate, mucus will begin to accumulate at the cell surface. Do not disturb the mucus layer.
4. Cells will be fully differentiated and ready for experimentation at **Day 21 ≅ ALI Day 14. NOTE:** If cells "Go to Air" early, they must still be kept at ALI for 14 days prior to experimentation.

P50:50 Base Media with Additives ≅ Porcine Complete Media Components

Supplement/Additive	Stock Conc.	Volume	Final Conc. in 500 ml
Nystatin (in-house, -20°C)	**10,000 U/ml**	**5 ml**	**100 U/ml**
Penicillin/Streptomycin Mix, -20°C	**10,000U/10mg/ml**	**5 ml**	**100U/0.1mg/ml**
Amphotericin B, -20°C	**250 µg/ml**	**2 ml**	**1 µg/ml**
Epidermal Growth Factor (EGF), -20°C	**5 µg/ml**	**500 µl**	**5 ng/ml**

Hydrocortisone, -20°C	**0.5 mg/ml**	**500 µl**	**0.5 µg/ml**
Transferrin, -20°C	**5 mg/ml**	**500 µl**	**5 µg/ml**
Insulin, -20°C	**5 mg/ml**	**500 µl**	**5 µg/ml**
Triiodothyronine (T3), -20°C	**20 µg/ml**	**500 µl**	**20 ng/ml**
Fetal Calf Serum (FCS), -70°C	**100%**	**10 ml**	**2%**
Retinoic Acid, -70°C	**15 mg/ml**	**500 µl**	**15 ng/ml**

[0.1 mg/ml L-glutamine, final conc., if not present in medium]

Aliquots are prepared such that adding the entire volume of 1 tube/vial will yield the correct final concentration in 500 ml of P50:50 DMEM/Ham's F12 Media.

Making 0.02 N Acetic Acid

For Collagen-coating Airway Epithelial Cell Culture Surfaces

1 ml Glacial Acetic Acid

870 ml dH_2O

Mix well, filter (0.2 □m) and store 4°C in a sterile (*i.e., autoclaved*) glass bottle.

KEEP STERILE!

Protocol # 7: Preparation of Swine Unit Dust Extract for *In Vitro* and *In Vivo* Use

Collecting Swine Unit Dust

1. Scraped or brushed settled dust from raised surfaces into zip lock bags. Collected several grams
2. Secure bags and transport to the laboratory immediately for further processing.
3. Dust must be used the same day it was collected

Preparing Swine Unit Dust Extract

1. Added 1 gram of dust to 10 ml Hank's Balanced Saline Solution without calcium.
2. Vortexed the mixture for 1 minute.
3. Let stand at room temperature for one hour.
4. Centrifuged for 10 minutes at 5,000 rpm at room temperature.
5. Transferred supernatant to a new tube.
6. Centrifuged again according to step 4.
7. Sterilized the final supernatant by filtration (0.22μm filter size).
8. The dust was used immediately.

Protocol #8: Preparing Mammalian Whole Cell Extracts

1. Placed cells on ice. Labeled microfuge tubes and place on ice.
2. Removed media from cells by vacuum-aspirating and washed gently with 1 ml cold PBS containing Phosphatase Inhibitor (1 ml aliquots at -20°C)
3. While on ice, add 100-200 µl ice-cold "Extraction Buffer" to each well (6 well plate)
    a. To make Extraction Buffer:
        i. Make 1X Cell Signaling Lysis Buffer
            1. Obtain 10X CS LB Stock (500 aliquots at -20°C) dilute with 4.5 ml deionized water.
        ii. Added 870 µl 1X CS Lysis Buffer prepared from (i.) to 100 µl 10X Roche® Complete Protease Inhibitor Cocktail Tablet aliquots (100 µl aliquots stored at -20°C, labeled "PIT").
        iii. Added 970 µl Pre-Extration Buffer(ii.) to
            1. 10 µl Phosphatase inhibitor Cockail 1(20 µl stored at 4°C)
            2. 10µl protease inhibitor cocktail 1(20 µl stored at -20°C)
4. Rocked plates on ice and work one plate at a time.
5. Used a cell scraper to scrape wells to one side of the well.
6. Transferred this lysate to the adjacent well. (Clean cell scraper in between samples with 70% ethanol and wipe with a kimwipe)
7. Transferred lysate to appropriate tube and return to ice
8. Sonicated 3 times for 7 second bursts with 1 minute incubations on ice
9. Pelleted debris at 15,000 rpm at 4°C for 15 minutes
10. Transferred supernatant to a clean tube.
11. Determined protein concentration via Bradford Assay

Protocol #9: Western Blot

SDS-PAGE Analysis

1. Added sample buffer to each extract (1/4 volume of 4X or ½ of 2X Laemmli Sample Buffer). Generally used 30-60 µg/ lane of cell extract for mini gels.
2. Boiled samples for 5 minutes.
3. Loaded samples onto SDS-PAGE gel.
4. Ran gel at 115 volts for 1 hour & 20 minutes at RT. (Run gels according to manufacturer's instructions).

Semi-Dry Transfer

1. Soaked 2 pieces of extra thick filter paper and one piece of nitrocellulose in Western transfer Buffer.
2. Made a sandwich (bottom to top) with 1 piece of filter paper, nitrocellulose, gel and 1 piece of filter paper. Place in semi-dry transfer apparatus and run at 10 V for 30-60 minutes.

Wet Tank Transfer

1. Presoaked 2 pieces of extra thick filter paper and one piece of nitrocellulose in ice cold transfer buffer.
2. Made sandwich with 1 filter pad, 1 piece of filter paper, nitrocellulose, gel, 1 piece of filter paper, 1 filter pad. Load into cassette and then into tank. Distribute ice around tank to keep temperature cool. Run gel at 100 V for 60 min.

Blocking

1. Nitrocellulose was removed from sandwich.
2. Blot was placed in 5% milk blocking solution and rocked at room temperature for one hour.
3. Blot was washed with TBS-T for 15 minutes with gentle agitation and washed twice for 5 minutes with gentle agitation.

Primary Antibody

1. Diluted appropriate antibody in TBS-T plus 0.5% BSA. Generally used 1:1000 (i.e. 10 µl antibody in 10 ml TBS-T/albumin).
2. Incubate blot overnight at 4ºC with agitation.
3. Blot was rinsed the following morning with TBS-T for 15 minutes once and washed twice for 5 minutes with TBS-T.

Secondary Antibody

1. Diluted secondary antibody in TBS-T plus 0.5% BSA. Generally used 5µl in 10 ml.
2. Incubated blot for one hour with agitation at room temperature.
3. Blot was washed with TBS-T for 15 minutes with gentle agitation and washed twice for 5 minutes with gentle agitation.

ECL Detection

1. Mixed equal amounts of ECL detection reagents (1-2 ml each per blot). It should come to room temperature before use.
2. Pipetted reagent over entire surface of membrane. Incubated for one minute.
3. Wicked excess liquid off by using dry edge of kimwipe.
4. Membrane was placed in sheet protector.
5. Exposed film to membrane. Exposure times vary depending on antibody and protein source.

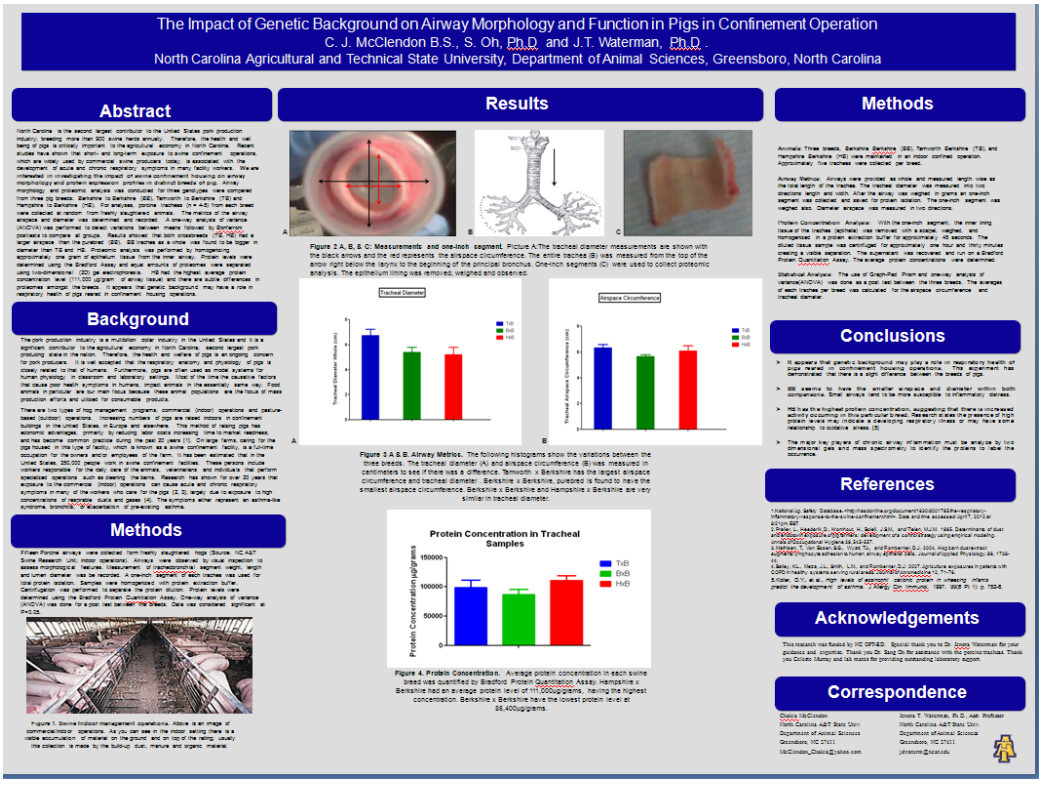
Manual Film Development

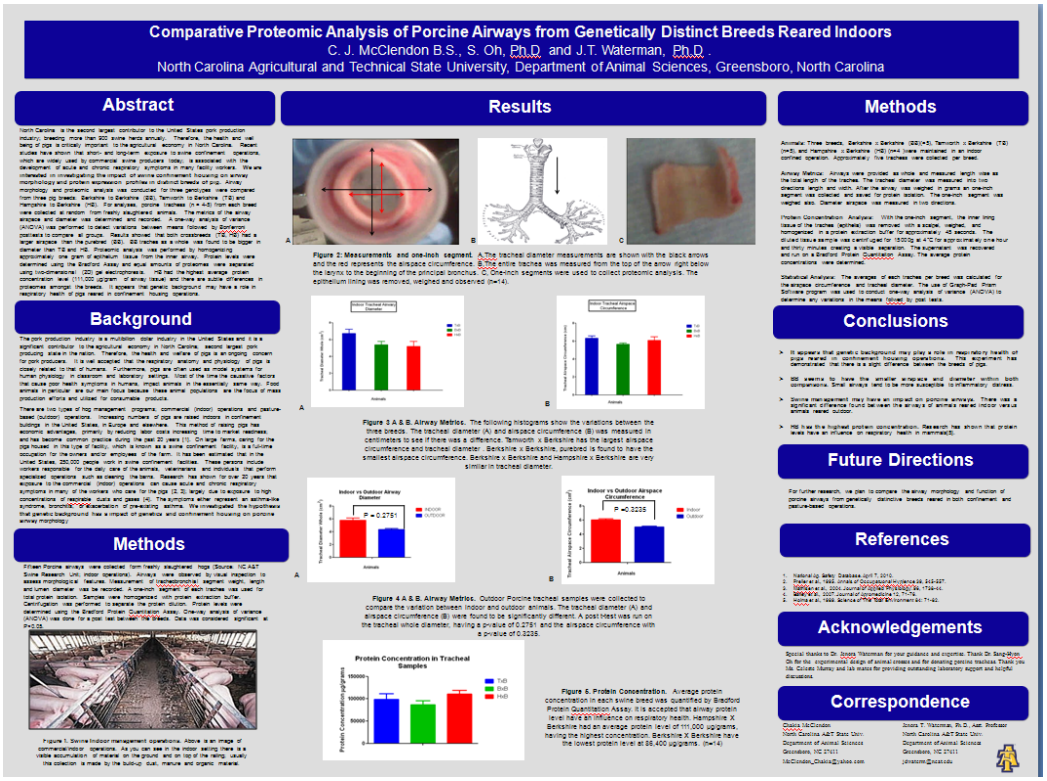
1. Made developer and fixer. After use wrap container in foil.
    a. Developer
        i. 800 ml warm tap water
        ii. 200 ml developer
        iii. Pour into flask and swirl
    b. Fixer
        i. 720 ml warm tap water (16ºC to 27ºC)
        ii. 250 ml rapid fixer (solution A)
        iii. 30 ml hardener (solution B)
        iv. Pour into flask and swirl

2. After exposure, film was placed in developer with agitation for 5 minutes.
3. Film was ran through warm running water bath.
4. Placed in fixer for 5 minutes with agitation.
5. Immediately film is placed in warm running water bath for 10 minutes.

Appendix B

Posters and Title Pages from Presentations





Comparative Proteomic Analysis of Porcine Airways from Genetically Distinct Breeds Reared Indoors

C. J. McClendon B.S., S. Oh, Ph.D. and J.T. Waterman, Ph.D.
North Carolina Agricultural and Technical State University, Department of Animal Sciences, Greensboro, North Carolina

Abstract

Background

Methods

Results

Methods

Conclusions

Future Directions

References

Acknowledgements

Correspondence

THE IMPACT OF SWINE MANAGEMENT OPERATIONS ON LARGE AIRWAY MORPHOLOGY OF PIGS

C. McClendon, S.-H. Oh and J.T. Waterman
North Carolina A&T State University
Department of Animal Sciences
ASAS Southern Section, Birmingham, Alabama
February 6, 2012